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In vitro Propagation to Conserve the Local Endemic and Endangered Medicinal Plant Helianthemum germanicopolitanum Bornm.

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HIGHLIGHTS

- This is the first report of the in vitro propagation of *Helianthemum germanicopolitanum* Bornm.
- It is a comprehensive optimization study in which 3 different media, 64 different PGR combinations and 2 different solidifiers are tested.
- The success of acclimatization is 32%.

Abstract: In this study, in vitro propagation and acclimatization of *Helianthemum germanicopolitanum* Bornm. plant, a local endemic in Çankırı Province (Turkey) with arid and semi-arid lands, and an endangered species taking part among medicinal and aromatic plants were accomplished, which is under-researched. In this study, three basal media [a) Murashige and Skoog b) Gamborg's B5, and c) Nitsch & Nitsch], two gelling agents (agar 7 g/L, and gelrite 2.1 g/L), eight cytokinins and eight auxin doses of plant growth regulators [a) 6-benzyladenin, b) Kinetin-(0, 0.5, 1, and 2 mg/L), c) Indole-3-butyric acid, d) α-napthaleneacetic acid-(0, 0.25, 0.5, and 1 mg/L)] prepared in 64 different combinations with 30 g/L sucrose was added to the basal media and adjusted to pH 5.7 for in vitro propagation of *H. germanicopolitanum*. During in vitro propagation of the plant, external and internal infections were frequently encountered and this was solved by the developed protocol. The best shoot growth (1.141 cm) and shoot length (0.572 cm) were obtained in the Gamborg's B5 medium in combination with Kinetin (0.5 mg/L)+Indole-3-butyric acid (0.5 mg/L)+gelrite. The maximum number of shoots (19.50) and the best multiplication rate (94%) were obtained in the media containing benzyladenin (1 mg/L)+Indole-3-butyric acid (0.5 mg/L) plant growth regulator in Murashige and Skoog medium solidified with agar. At the rooting stage, the maximum number of roots (30) was reached in the Murashige and Skoog medium containing gelrite and the best rooting rate (92%) with agar. A hundred plants representing the best shoot and root growth were taken to acclimatization stage, and 32 of these plants adapted to external conditions.

Keywords: acclimatization; *Helianthemum germanicopolitanum;* internodes; micropropagation; risk of extinction.

INTRODUCTION

Excessive urbanization by the construction industry and the expansion of agricultural lands has caused rapid destruction of the natural areas in recent years. Plant and animal species living in the natural areas are under the threat of extinction due to this destruction. The local endemic plant *Helianthemum germanicopolitanum* Bornm., a medicinal and aromatic plant under the threat of extinction, grows in Çankırı province, Turkey (Figure 1). *H. germanicopolitanum* in the Cistacea family is protected by organizations, such as the International Union for Conservation of Nature (IUCN), Threatened Plant Committee (TPC), and World Wildlife Foundation (WWF), as well as by the Turkish Ministry of Agriculture and Forestry as an endangered (EN) plant.

Figure 1. *H. germanicopolitanum* Bornm.'s **a)** flowers, **b-c)** habitus and **d)** habitat.

It is significant to protect some endemic species in their natural habitats and ensure their propagation. In addition to this, it is necessary to perform the cultivation of these plants quickly. The collection of propagated plants and ensuring their continuity *in vitro* is of considerable importance for the presence of the plant at the risk of extinction. To protect the soil within the scope of combating desertification, the *Helianthemum* sp., which has very important effects on keeping the elements that play a vital role in plant nutrition, such as N, P, K, in the soil, should be protected. In Turkey, Çankırı is located in the arid and semi-arid zones and began to gain similar characteristics to the desert ecosystem increasingly concerning climate, water regime, soil, and vegetation, [1]. In this sense, the preservation of ecological features will be provided primarily by preserving the soil in place. It is obvious that the *Helianthemum* sp. is a very precious plant with its well-developed root structure and adaptation to less rainy climatic conditions [2].

Helianthemum sp. is an important herb with many medicinal properties. This plant has been used in the treatment of digestive system disorders, such as diarrhea, bloody diarrhea, abdominal and epigastric pain, since the epoch of Maya's [3]. Essential oil, tannin and mucilage of *H. germanicopolitanum* have diuretic and constipation relieving effects, as well as it is particularly used effectively against hemorrhoids in Turkey. In addition, *Helianthemum* species have been used worldwide for the treatment of gastrointestinal disorders, wounds and burns with its antidiarrheal, anti-inflammatory, antiulcerogenic, antiparasitic, antimicrobial, analgesic, vasodilating [4,5]. In addition, leaves and stems of *H. syriacum* (Jacq.) Dum. Cours. species are consumed in drinks in Spain [6].

Antiprotozoal, antigiardial, and anti-inflammatory effects of *H. glomaretaum* (Lag.) Lag. ex Dunal species [7,8], antiamibic property of *H. lippi* (L.) species [9], the antimalarial effect of *H. ventosum* Boiss. species [10], antibacterial, antioxidant properties of *H. ledifolium* L. species [11,12], and the antimicrobial effect of *H. kahiricum* Del. [9] have been studied. In essential oil analysis of the flowering aerial parts of the *H. canum* (L.) Baumg. species, myristicin (29.4%), T-cadinol (6.5%), hexadecanoic acid (5.2%) and spatulenol (3.6%) were identified as the main compounds [13,14]. As the endemic species are propagated by seed [15], their propagation by tissue culture method has an important place in terms of the protection of the species and their rapid and clonal production. The propagation of *H. germanicopolitanum in vitro* has not been studied yet.

In the present study, the effects of both plant growth regulators (PGRs) and media combinations on the shoot, callus and root formation were investigated by cultivating *H. germanicopolitanum* explants and propagation *in vitro* by tissue culture method for the first time, using three different media, 64 different PGR combinations and two different solidifiers.

MATERIAL AND METHODS

Plant materials and sterilization

H. germanicopolitanum samples were collected on the side of the Karakışla road, on the upper parts of Çakmaklıdere valley, on gypsum hills at the altitude of 650-800 m at 40º29'30'' N - 033º39'36'' E coordinates, and on the Çankırı-Kalecik-İnandık road turnout, located in gypsum and limy hills at an altitude of 831 m in 40º23'15'' N -033º35'01'' E coordinates (Figure 2) [16,17,18]. In addition, it spreads in Çankırı Kenbağ area, Çankırı Esentepe neighborhood and between Esentepe-Doğantepe roads.

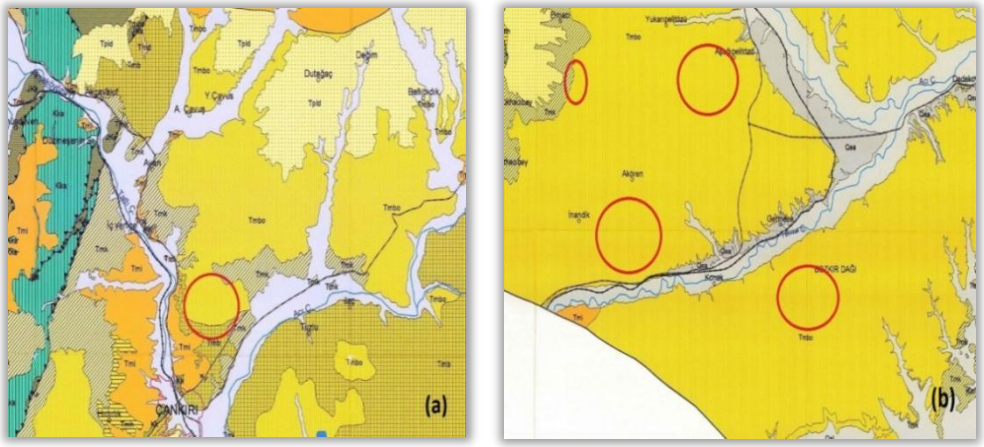

Figure 2. Land and geological formation views of *H. germanicapolitanum* **a)** Çankırı Kenbağ area **b)** Karakışla Village Road Çakmaklıdere Valley and İnandık Village

Plants were collected as a limited amount of shoots in June, July and August 2018, in a way that will not harm the environment. The plant samples were washed 30 min under running tap water, and treated with 1 g/L Cuprosan GW- (Bayer®) for 20 min. Then, the plants were treated with 1 g/L $HgCl_2$ 15 min and 70% EtOH 10 min. After the leaves on the plants were cleaned, they were cut at least 2 cm in length and at least two buds on them. To increase the efficiency of chlorine, 1-2 drops of Tween 20 was added to the 30% concentration of commercial NaOCl containing 15% chlorine by volume and applied for 15 min. Lastly, the plants were rinsed for five min for three times with sterile distilled water and dried under aseptic conditions.

Media Preparation and Shoot Regeneration

Micropropagation method was applied using shoot tip internodes and nodes. The node and shoot tips with at least two buds on them were removed from the leaves of the *H. germanicopolitanum* samples, and planted in De-Wit culture tubes after the sterilization phase as an explant source. At micropropagation initial stage, three different basal media, Murashige and Skoog (MS-macro and micro elements including vitamins, Duchefa®) [19], Gamborg's B5 (macro and micro elements including vitamins, Duchefa®) [20], and Nitsch & Nitsch (N&N-macro and micro elements including vitamins, Duchefa®) [21] and solidifiers, agar (7 g/L-Duchefa®) and gelrite (2.1 g/L-Duchefa®) [22] were used. The PGRs, a) 6-benzyladenin (BA-Duchefa®) (0, 0.5, 1, and 2 mg/L), b) Kinetin (KIN-Duchefa®) (0, 0.5, 1, and 2 mg/L), c) Indole-3-butyric acid (IBA- Duchefa®)

(0, 0.25, 0.5, and 1 mg/L), and d) α-napthaleneacetic acid (NAA-Duchefa®) (0, 0.25, 0.5, and 1 mg/L) were prepared in 64 different combinations with 30 g/L sucrose, added to the media, and adjusted to pH 5.7 for *in vitro* propagation of *H. germanicopolitanum.* Three basal media, two solidifiers, eight cytokinin doses (BA and KIN 0, 0.5, 1, 2 mg/L) and eight auxin doses (IBA and NAA 0, 0.25, 0.5, 1 mg/L) and 30 g/L sucrose were used to obtain 384 different combinations, and then the pH of the media was adjusted to 5.7. Plant Preservative Mixture (PPM, Duchefa®) was added as 1 mL/L to prevent contamination that may occur after planting explants in the media at every stage [23]. All combinations were prepared in 10 repetitions, with one explant per tube (Dewit-Duchefa® 130x10 mm). Within the scope of regeneration, all cultures were incubated for four weeks in a growth chamber with a temperature of 25±1ºC and 16 h of light (day-light fluorescent lamps (Philips®) at a photon flux density of 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$), eight h of dark conditions, and checked regularly every day. In this method, different compositions of media were used in the initial stage. At this stage, the effects of media, solidifier type and PGR combinations were investigated.

Sub-culture

The explants were transferred on MS, Gamborg's B5, and N&N media, depending on the combinations of PGR that were the same as the initial stage. Following the initial stage, subcultures were performed three times with an interval of four weeks to enable the explants to develop shoot and root. Subculture media contains; initial stage media, including PGR combinations and solidifiers. At this stage of development, explants showing direct shoot growth were transferred to rooting media.

Rooting stage

Regenerated shoots were cultured in same media containing auxin group PGRs [Indol-3-butyric acid (IBA) (0, 0.25, 0.5, and 1 mg/L) α-naphthalenacetic acid (NAA) (0, 0.25, 0.5, and 1 mg/L)] with agar or gelrite. Plantings were made in sterile containers (ECO2 BOX-Duchefa® 125x65x80 mm). Plants were kept in the rooting medium for four weeks. During the rooting stage, all cultures were stored in a growth chamber with a temperature of 25±1ºC, and 16h-photoperiod.

Acclimatization

Plantlets with good roots and shoot growth were thoroughly washed under distilled water to remove the remaining medium and agar. These plants were then planted in 10x8 cm plastic pots containing an autoclaved mixture of garden soil, sand and perlite (3:1:1 v/v). The watered plants were covered with plastic bags and small air vents were opened to prevent moisture loss. These plants were incubated in a humidity-controlled growth chamber at 25±1 °C in 16 h light conditions. The plants were acclimatized between four and six weeks, and the plastic bags on the pots were completely opened gradually. After planting, the plants were watered using approximately 50 mL of tap water every other day.

Statistical Analyses

All of the experiments in this study were performed according to the "Randomized complete block design". The effects of three different applications (3 basal media x 64 PGR combinations x 2 solidifiers) in the shoot formation experiments were investigated and each experiment was set up as 10 explants (3840 internodes). During the rooting, a basal medium with eight doses of two PGRs were studied and 10 shoots were used in each trial. All the experiments were repeated for two years.

All data (shoot length and number, root length and number) were analyzed using SPSS® 20.0 (IBM Corporation software), and analysis of variance (ANOVA) was carried out using a three-factor randomized complete plot design. Significant F-values ($P \leq 0.05$, 0.01, and 0.001) were calculated as differences between individual means using the Duncan test.

RESULTS AND DISCUSSION

The shoot, node and apical parts of the plant were successfully used as an initial explant in tissue culture of *Helianthemum* sp. genera, as well as in many plants [24, 25,26,27,28,29]. In the present study, depending on the development of explants, the media in which they were planted and the PGR combinations they contain, callus, shoot and root formations were observed. In every subculture experiment carried out after the initial phase, the number of shoots and roots of *H. germanicopolitanum* were determined.

Initial Shoot Length

In *H. germanicopolitanum* explants, higher shoots at the initial stage were obtained in the combination of “basal media x PGR combination x solidifier” Gamborg's B5 medium, KIN (0.5 mg/L) + IBA (0.5 mg/L) combination and in the medium that was solidified with the gelrite (Figure 3g). Besides, the shoot length were well observed in the medium that contains KIN (1 mg/L) + IBA (0.25 mg/L) combination and solidified with gelrite (Table 1). Serrano-Martinez and coauthors in *H. marminorense*, Hamza and coauthors and Salih and coauthors in *H. lippii* L. var. *sessiliforium*, [26, 27, 29] used only MS and DKW basal media. In our study, an optimization method was applied for *in vitro* propagation of *H. germanicopolitanum* using three different basal media together with PGR combinations.

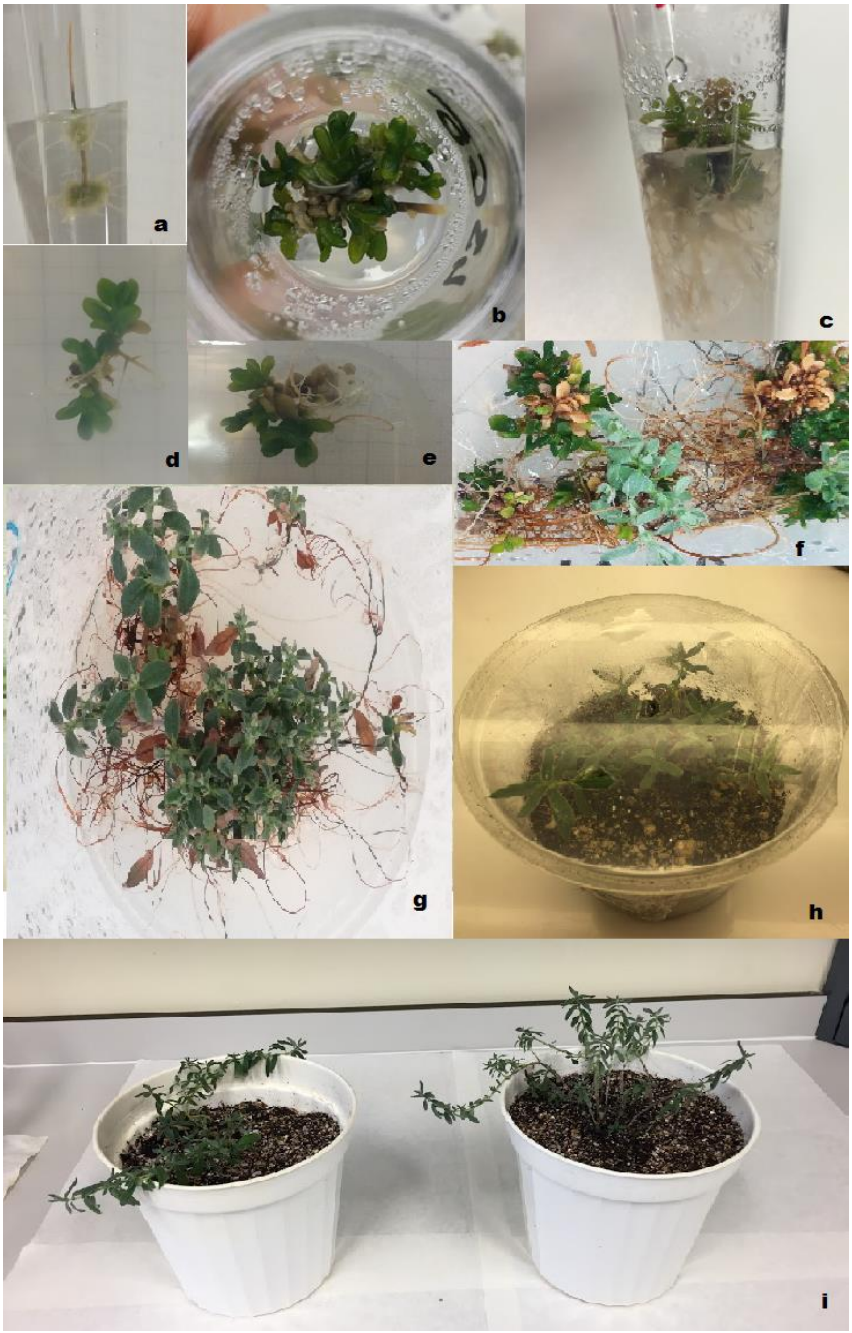

Figure 3. Micropropagation stages of *H. germanicopolitanum* **a)** Rooting in N&N medium with BA (0 mg/L)+NAA (1 mg/L) and gelrite **b)** Shoot growth in MS medium with BA (1 mg/L)+IBA (0.5 mg/L) and agar **c)** Initial shoot and rooting stages in Gamborg’s B5 medium with BA (0.5 mg/L)+IBA (0 mg/L) and agar **d)** Initial shoot and rooting stages in N&N medium with IBA (0.5 mg/L) and agar **e)** Initial shoot and rooting stages in MS medium with BA (1 mg/L) + IBA (0.5 mg/L) and agar **f)** *H. germanicopolitanum* that has completed shoot and root development in N&N medium with IBA (1mg/L) and gelrite **g)** Plant regeneration KIN (0.5 mg/L) + IBA (0.5 mg/L) and gelrite containing Gamborg’s B5 medium **h)** Acclimatization stage of regenerated plants **i)** The plants adapted to external conditions.

Table 1. The effect of the interaction between the "basal media x PGR combination x solidifier" of shoot length (cm) after the initial phase in *H. germanicopolitanum* explants (60th day) (P≤0.001).

	PGR Combinations (mg/L)				MS		Gamborg's B5		N&N	
	BA	KIN	IBA	NAA	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite
1	0	0	0	0	0(i)*	0	0	0(i)	0(i)	0(i)
2	0	0	0.25	0	0.30(ghi)	0	0	0	0	0
3	0	0	0.5	0	0	0.01(i)	0	0	0	0
4	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0.15(i)	0	0	0	0
6	0	0	0	0.25	0	0	0	0	0	0
7	0	0	0	0.5	0	0	0	0.05(hi)	0	0
8	0	0	0	1	0.20(hi)	0	0	0	0	0.05(hi)
9	0.5	0	0	0	0	0	0.15(i)	0	0	0
10	0.5	0	0.25	0	0	0	0	0	0	0
11	0.5	0	0.5	0	0	0	0.16(i)	0.10(i)	0	0
12	0.5	0	1	0	0	0	0	0	0.20(hi)	0
13	0.5	0	0	0	0	0.21(i)	0	0	0	0
14	0.5	0	0	0.25	0	0	0	0	0	0
15	0.5	0	0	0.5	0	0	0	0.03(i)	0	0
16	0.5	0	0	1	0	0	0	0	0	0
17	1	0	0	0	0	0	0	0	0	0
18	1	0	0.25	0	0	0.16(i)	0.18(i)	0	0	0
19	1	0	0.5	0	0.50(cde)	0	0.49(cde)	0	0	0
20	1	0	1	0	0.02(i)	0	0.05(hi)	0	0	0
21	1	0	0	0	0	0	0	0.10(i)	0	0
22	1	0	0	0.25	0.01(i)	0.05(hi)	0	0	0	0
23	1	0	0	0.5	0	0	0	0	0	0
24	1	0	0	1	0	0	0	0	0	0
25	2	0	0	0	0	0	0	0	0	0.03(i)
26	2	0	0.25	0	0	0	0	0	0.05(hi)	0
27	2	0	0.5	0	0.04(hi)	0	0	0	0	0
28	2	0	1	0	0	0	0	0	0	0
29	2	0	0	0	0	0	0	0	0	0
30	2	0	0	0.25	0	0	0	0	0	0
31	2	0	0	0.5	0	0	0	0	0	0
32	2	0	0	1	0	0	0	0	0	0.03(i)
33	0	0	0	0	0	0	0	0	0	0
34	0	0	0.25	0	0	0	0	0	0	0
35	0	0	0.5	0	0	0.25(ghi)	0	0	0	0
36	0	0	1	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0.25	0	0	0	0	0	0
39	0	0	0	0.5	0	0	0	0	0	0.03(i)
40	0	0	0	1	0	0	0	0	0	0
41	0	0.5	0	0	0	0	0	0	0	0
42	0	0.5	0.25	0	0	0	0	0	0	0
43	0	0.5	0.5	0	0	0	0	**1.14(a)**	0	0.09(hi)
44	0	0.5	1	0	0	0	0	0	0	0
45	0	0.5	0	0	0.25(ghi)	0	0	0	0	0
46	0	0.5	0	0.25	0	0	0	0	0	0
47	0	0.5	0	0.5	0	0	0	0	0	0
48	0	0.5	0	1	0	0	0	0	0	0
49	0	1	0	0	0	0	0	0	0	0
50	0	1	0.25	0	0	0	0	**0.84(ab)**	0	0
51	0	1	0.5	0	0	0.65(bc)	0	0.43(def)	0	0
52	0	1	1	0	0.07(hi)	0	0	0.36(fghi)	0	0
53	0	1	0	0	0	0	0	0.25(ghi)	0	0.35(fghi)
54	0	1	0	0.25	0	0	0	0.75(bc)	0	0.05(hi)
55	0	1	0	0.5	0	0	0	0	0	0
56	0	1	0	1	0	0.05(hi)	0	0.14(hi)	0	0.03(i)
57	0	2	0	0	0	0	0	0.22(ghi)	0	0.36(efg)
58	0	2	0.25	0	0.06(hi)	0	0	0	0.45(cde)	0
59	0	2	0.5	0	0	0	0	0	0	0
60	0	2	1	0	0	0	0	0	0	0.05(hi)
61	0	2	0	0	0	0.09(hi)	0	0	0	0
62	0	2	0	0.25	0	0	0	0.47(cde)	0.05(hi)	0.02(i)
63	0	2	0	0.5	0.07(hi)	0	0.52(cd)	0	0	0.05(hi)
64	0	2	0	1	0	0	0.28(ghi)	0	0	0

*Different letters indicate significant differences between all the treatments

Initial Shoot Number

In the *in vitro* propagation of *H. germanicopolitanum*, the number of shoots was recorded after the initial stage, and the data obtained were evaluated statistically. The effect of “basal media x PGR combination x solidifier” interaction was found statistically significant (P≤0.001). In MS medium with BA (1 mg/L) + IBA (0.5 mg/L) and agar (the number of shoots 19.50), and the best shoot number (19.06) was recorded when KIN (2 mg/L) + IBA (0 mg/L) combination was used in N&N medium and gelrite (P≤0.05) (Table 2, Figures 3b-3e). Additionally, in both MS medium BA (0.5 mg/L) + NAA (0 mg/L) combination and gelrite (18.10), and N&N medium KIN (2 mg/L) + IBA (0.25 mg/L) combination and agar (18.00) the number of shoots was found statistically significant (Table 2). In *H. germanicopolitanum* explants propagated by direct organogenesis, the best multiplication rate (94%) was obtained with MS medium with agar, while this ratio (88%) was obtained with N&N medium with gelrite. Serrano-Martinez and coauthors (2012) reported that 0.5 mg/L 2iP containing the WPM medium provided the best shoot number in *H. marminorense* [26], Hamza and coauthors (2013) found maximum shoot number and shoot development for *H. lippii* L. var. *sessiliforium* in the MS medium containing 0.5 and 1.0 mg/L BA [27], and Salih and coauthors (2019) studied the same species reporting that the highest shoot development occurred in different doses (0.5-1.0-1.5-2.0 mg/L) of BA, which was also cultured in the MS medium [29].

Table 2. The effect of the interaction between the "basal media x PGR combinations x solidifier" of shoot numbers after the initial phase in *H. germanicopolitanum* explants (P≤0.001).

	PGR Combinations (mg/L)				MS		Gamborg's B5		N&N	
	BA	KIN	IBA	NAA	Agar	Gelrite	Agar	Gelrite	Agar	Gelrite
1	0	0	0	0	0	0	0	0	0(i)	0(i)
2	0	0	0.25	0	0.50(hi)*	0	0	0	0	0
3	0	0	0.5	0	0	0.50(hi)	0	0	0	0
4	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	1.50(ghi)	0	0	0	0
6	0	0	0	0.25	0	0	0	0	0	0
7	0	0	0	0.5	0	0	0	0.30(hi)	0	0
8	0	0	0	1	2.40(ghi)	0	0	0	0	0.10(i)
9	0.5	0	0	0	0	0	3.00(fgh)	0	0	0
10	0.5	0	0.25	0	0	0	0	0	0	0
11	0.5	0	0.5	0	0	0	3.10(fgh)	0.50(hi)	0	0
12	0.5	0	1	0	0	0	0	0	10.00(defg)	0
13	0.5	0	0	0	0	**18.10(ab)**	0	0	0	0
14	0.5	0	0	0.25	0	0	0	0	0	0
15	0.5	0	0	0.5	0	0	0	0.10(i)	0	0
16	0.5	0	0	1	0	0	0	0	0	0
17	1	0	0	0	0	0	0	0	0	0
18	1	0	0.25	0	0	11.80(def)	0.80(hi)	0	0	0
19	1	0	0.5	0	**19.50(a)**	0	2.00(gh)	0	0	0
20	1	0	1	0	0.10(i)	0	0.40(hi)	0	0	0
21	1	0	0	0	0	0	0	0.20(i)	0	0
22	1	0	0	0.25	1.50(ghi)	0.10(i)	0	0	0	0
23	1	0	0	0.5	0	0	0	0	0	0
24	1	0	0	1	0	0	0	0	0	0
25	2	0	0	0	0	0	0	0	0	0.50(hi)
26	2	0	0.25	0	0	0	0	0	1.00(hi)	0
27	2	0	0.5	0	0.40(hi)	0	0	0	0	0
28	2	0	1	0	0	0	0	0	0	0
29	2	0	0	0	0	0	0	0	0	0
30	2	0	0	0.25	0	0	0	0	0	0
31	2	0	0	0.5	0	0	0	0	0	0
32	2	0	0	1	0	0	0	0	0	0.10(i)
33	0	0	0	0	0	0	0	0	0	0
34	0	0	0.25	0	0	0	0	0	0	0
35	0	0	0.5	0	0	1.70(ghi)	0	0	0	0
36	0	0	1	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0.25	0	1.00(hi)	0	0	0	0
39	0	0	0	0.5	0	0	0	0	0	0.20(i)
40	0	0	0	1	0	0	0	0	0	0
41	0	0.5	0	0	0	0	0	0	0	0
42	0	0.5	0.25	0	0	0	0	0	0	0
43	0	0.5	0.5	0	0	0	0	2.35(ghi)	0	15.38(bc)
44	0	0.5	1	0	0	0	0	0	0	0
45	0	0.5	0	0	13.50(cdef)	0	0	0	0	0
46	0	0.5	0	0.25	0	0	0	0	0	0
47	0	0.5	0	0.5	0	0	0	0	0	0
48	0	0.5	0	1	0	0	0	0	0	0
49	0	1	0	0	0	0	0	0	0	0
50	0	1	0.25	0	0	0	0	3.84(fgh)	0	0
51	0	1	0.5	0	0	9.27(de)	0	8.30(efgh)	0	0
52	0	1	1	0	0	0	0	1.78(ghi)	0	0
53	0	1	0	0	0	0	0	0.50(hi)	0	14.50(cde)
54	0	1	0	0.25	0	0	0	1.40(ghi)	0	16.50(bc)
55	0	1	0	0.5	0	0	0	0	0	0
56	0	1	0	1	0	0.10(i)	0	0.70(hi)	0	14.00(cde)
57	0	2	0	0	0	0	0	0.20(i)	0	**19.06(a)**
58	0	2	0.25	0	13.50(cdef)	0	0	0	**18.00(ab)**	0
59	0	2	0.5	0	0	0	0	0	0	0
60	0	2	1	0	0	0.40(hi)	0	0	0	0.20(i)
61	0	2	0	0	0	4.50(fghi)	0	0	0	0
62	0	2	0	0.25	0	0	0	3.27(fgh)	8.00(efgh)	0.10(i)
63	0	2	0	0.5	0.30(hi)	0	1.90(gh)	0	0	0.20(i)
64	0	2	0	1	0	0	3.40(fg)	0	0	0

*Different letters indicate significant differences between all the treatments

Initial Root Number and Length

In *H. germanicopolitanum* explants, the effect of the "basal media x PGR combination x solidifier" interaction was found to be significant (P≤0.05) as the best root number (0.5) and root length in the N&N medium containing BA (0 mg/L) + NAA (1 mg/L) solidified with the gelrite (Table 3, Figure 3a). While Gamborg's B5 medium with the agar was found to be important concerning the number of roots (root number 0.4) (Table 3, Figure 3c). In addition to being a local endemic species living on a salty and gypsiferous soil, *H.germanicopolitanum*'s woody structure has been quite challenging and restrictive in the in vitro rooting stage. In terms of root number, obtaining such low numbers in statistical terms can be explained by this reason. Gamborg's B5 medium, which has more average amounts of potassium and ammonium than other media in rooting, has shown its effect here. In a micropropagation study for *H. lippii* L. var *sessiliforium*, the best root growth and the highest root length were obtained on PGR-free MS medium [27]. Serrano-Martinez and coauthors (2012) used *H. marminorense* and observed the best root number and root development in the WPM medium without PGR [26]. Hamza and coauthors (2014) observed a significant growth in MS medium containing 1 mg/L IBA and MS medium with 1 mg/L NAA for *H. kahiricum* [28].

Table 3. The effect of the interaction between the "basal media x PGR combinations x solidifier" of the root number at the initial stage in *H. germanicopolitanum* explants (P≤0.001).

	PGR Combinations (mg/L)				MS		Gamborg's B5		N&N	
	BA	**KIN**	**IBA**	**NAA**	**Agar**	**Gelrite**	**Agar**	**Gelrite**	**Agar**	**Gelrite**
1	0	0	0	0	0(g)	0(g)	0	0	0	0
2	0	0	0.25	0	0	0	0	0	0	0
3	0	0	0.5	0	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0.25	0	0	0	0	0	0
7	0	0	0	0.5	0	0	0	0	0	0
8	0	0	0	1	0	0	0	0	0	**0.50(a)**
9	0.5	0	0	0	0	0	**0.40(ab)**	0	0	0
10	0.5	0	0.25	0	0	0	0	0	0	0
11	0.5	0	0.5	0	0.10(cef)	0	0	0	0	0
12	0.5	0	1	0	0	0	0	0	0	0
13	0.5	0	0	0	0	0	0	0	0	0
14	0.5	0	0	0.25	0	0	0	0	0	0
15	0.5	0	0	0.5	0	0	0	0	0	0
16	0.5	0	0	1	0	0	0.10(cef)	0	0	0
17	1	0	0	0	0	0	0	0	0	0
18	1	0	0.25	0	0	0	0	0.10(cef)	0	0
19	1	0	0.5	0	0	0	0	0	0	0
20	1	0	1	0	0	0	0	0	0	0
21	1	0	0	0	0	0	0	0	0	0
22	1	0	0	0.25	0	0	0	0	0	0
23	1	0	0	0.5	0	0	0	0	0	0
24	1	0	0	1	0	0	0	0	0	0
25	2	0	0	0	0	0	0	0	0	0
26	2	0	0.25	0	0	0	0	0	0.20(bce)	0
27	2	0	0.5	0	0	0	0	0	0	0
28	2	0	1	0	0	0	0	0	0	0
29	2	0	0	0	0	0	0	0	0	0
30	2	0	0	0.25	0	0	0	0	0	0
31	2	0	0	0.5	0	0	0	0	0	0
32	2	0	0	1	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0
34	0	0	0.25	0	0	0	0	0	0	0
35	0	0	0.5	0	0	0	0	0	0	0
36	0	0	1	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0.25	0	0	0	0	0	0
39	0	0	0	0.5	0	0	0	0	0	0
40	0	0	0	1	0	0	0	0	0	0
41	0	0.5	0	0	0	0	0	0	0	0
42	0	0.5	0.25	0	0	0	0	0	0	0
43	0	0.5	0.5	0	0	0	0	0	0	0.07(cef)
44	0	0.5	1	0	0	0	0	0	0	0
45	0	0.5	0	0	0	0	0	0	0	0
46	0	0.5	0	0.25	0	0	0	0	0	0
47	0	0.5	0	0.5	0	0	0	0	0	0
48	0	0.5	0	1	0	0	0	0	0	0
49	0	1	0	0	0	0	0	0	0	0
50	0	1	0.25	0	0	0	0	0	0	0
51	0	1	0.5	0	0	0	0	0	0	0
52	0	1	1	0	0	0	0	0	0	0
53	0	1	0	0	0	0	0	0	0	0
54	0	1	0	0.25	0	0	0	0	0	0
55	0	1	0	0.5	0	0	0	0	0	0
56	0	1	0	1	0	0	0	0	0	0
57	0	2	0	0	0	0	0	0	0	0
58	0	2	0.25	0	0	0	0	0	0	0
59	0	2	0.5	0	0	0	0	0	0	0
60	0	2	1	0	0	0	0	0	0	0
61	0	2	0	0	0	0	0	0	0	0.30(bc)
62	0	2	0	0.25	0	0	0	0	0	0
63	0	2	0	0.5	0	0	0	0	0	0
64	0	2	0	1	0	0	0	0	0	0

*Different letters indicate significant differences between all the treatments

Root Number in Rooting Stage

In the *in vitro* propagation of *H. germanicopolitanum*, the number of roots they formed in the rooting media was taken after the subculture stage and the obtained data were evaluated statistically. At this stage, the effects of IBA and NAA, dosage, medium, and the effects of the solidifiers were examined. Accordingly, the effect of the interaction between "PGR x PGR doses x basal media" and the interaction between "PGR doses x basal media x solidifier" were found statistically significant (P≤0.05). In addition to the effect of media in rooting, the auxin group of PGR used was effective. While 0.5 mg/L dose of PGRs gave positive results in all experiments (Table 4), the MS medium with gelrite also gave the best results concerning the number of roots (30.08) and the number of roots in Gamborg's B5 medium was 17.25. The use of 0.5 mg/L IBA added to Gamborg's B5 (number of roots: 16.25) and MS (number of roots: 15.0) media in rooting studies, and the use of gelrite as a solidifier were sufficient for successful rooting in *H. germanicopolitanum* (Table 5). In this context, the best rooting rate of the study (92%) was obtained in MS medium solidified with agar and (88%) was also obtained in N&N medium with gelrite. Root growth is generally suppressed by ammonium and promoted by nitrate, N&N medium contains a lower concentration of nitrate and salt than other media. Therefore, the success of MS and Gamborg's B5 is appeared due to this reason. The use of PGR free medium or, on the contrary, high concentration of auxin affects rooting success [30]. The 0.5 mg / L and 1 mg / L auxin doses used in the rooting studies of H.germanicopolitanum also yielded successful results in progressive subcultures. Hamza and coauthors (2013) added different doses of IBA and NAA to the MS medium in initial stage then achieved the highest rooting rate of 71.80% in MS PGR free medium in *H. lippii* L. var *sessiliflorum* [27]. Hamza & Neffati (2014) obtained the highest rooting (94 -98%) in *H. kahiricum* with the addition of 1 mg/L IBA and NAA to the ½ MS medium [28]. In *H. almeriense*, the sixth subculture achieved the highest rooting (92%) with a low cytokinin rate [24].

Table 4. The effect of interaction between "PGR doses x basal media x solidifier" of root numbers in rooting stage of *H. germanicopolitanum* explants (P≤0.05).

	Basal Media					
	MS		**Gamborg's B5**		**N&N**	
PGR Doses	**Agar**	**Gelrite**	**Agar**	**Gelrite**	**Agar**	**Gelrite**
0 mg/L	2.50(fghi)*	0(i)	0(i)	0(i)	0(i)	0(i)
0.25 mg/L	5.38(fg)	1.83(ghi)	0(i)	0(i)	4.25(fgh)	1.15(hi)
0.5 mg/L	1.25(hi)	**30.08(a)**	4.00(fgh)	17.25(b)	9.40(cde)	8.80(cde)
1 mg/L	0(i)	1.80(ghi)	4.27(fg)	10.83(cd)	11.65(cd)	12.41(cd)

*Different letters indicate significant differences between all the treatments

Table 5. The effect of interaction between "PGRs x PGR doses x Basal media" of root number in rooting stage of *H. germanicopolitanum* explants (P≤0.05).

	Basal Media					
	MS		**Gamborg's B5**		**N&N**	
PGR Doses	NAA	IBA	NAA	IBA	NAA	IBA
0 mg/L	5.00(de)*	2.25(efg)	0(g)	0(g)	0(g)	0(g)
0.25 mg/L	0.83(fg)	3.66(efg)	0(g)	0(g)	1.40(fg)	4.00(efg)
0.5 mg/L	3.42(efg)	**15.00(a)**	6.00(de)	**16.25(a)**	12.33(bc)	5.87(de)
1 mg/L	0(g)	1.80(fg)	5.06(de)	10.04(bc)	12.83(bc)	11.23(bc)

*Different letters indicate significant differences between all the treatments

Root Length in Rooting Stage

In *H. germanicopolitanum* explants, the interaction between "PGR x basal media" was found statistically significant (P≤0.05) for root lengths in the rooting stage (Table 6). The effect of IBA on root growth was more effective than NAA (Figure 3d). The maximum root length occurred in the Gamborg's B5 medium with 1,914 cm. The richness of Gamborg's B5 in terms of potassium and ammonium might provide a rooting enhancing effect. Hamza & Neffati (2014) also achieved the highest root length (200-450 mm) in ½MS medium with 1 mg/L IBA and NAA, later in PGR free MS medium in *H. kahiricum* [28].

Table 6. The effect of interaction between "PGR x basal media" of root length during rooting stage in *H. germanicopolitanum* explants (P≤0.05).

	Plant Growth Regulator (PGR)	
Basal media	**NAA**	**IBA**
MS	0.223(e)*	0.685(cde)
Gamborg's B5	0.538(de)	**1.914(a)**
N&N	0.749(bcd)	0.972(bc)

*Different lowercase letters indicate significant differences between all the treatments

Shoot Length and Number in Rooting Stage

The statistical analysis performed in the rooting stage of *H. germanicopolitanum* explants showed that the best shoot length were found in N&N medium (0.636), which contains gelrite as a solidifier (Table 7), and NAA had a better effect on shoot development than IBA (0.572). The N&N was the best medium in terms of shoot growth, and Gamborg's B5 medium provided at least as much growth as the N&N medium concerning shoot length.

Table 7. The effect of interaction between "PGR x basal media x solidifier" of shoot length during rooting stage in *H. germanicopolitanum* explants (P≤0.05).

	Basal Media					
	MS		**Gamborg's B5**		**N&N**	
PGR	**Agar**	**Gelrite**	**Agar**	**Gelrite**	**Agar**	**Gelrite**
IBA	0.312(def)*	0.513(bc)	0.046(f)	**0.572(ab)**	0.325(cdef)	0.416(bcd)
NAA	0.204(ef)	0.405(bcd)	**0.599(ab)**	0.367(cde)	0.313(def)	**0.636(a)**

*Different lowercase letters indicate significant differences between all the treatments

At this stage, the effects of IBA and NAA, amount of dosage, medium, and effects of solidifiers were examined. Accordingly, the effect of the "PGR x basal media x solidifier" interaction was found statistically significant (P≤0.05). N&N medium containing NAA and gelrite interaction (0.636) was significant (Figure 3f). The interaction between "basal media x solidifier" in the rooting stage of *H. germanicopolitanum* explants produced statistically the best results (P≤0.05) for shoot numbers in the Gamborg's B5 media where agar was used as a solidifier (Table 8) (29.842). Agar, a high molecular polysaccharide, generally affects growth and development compared to gelrite [30]. Gelrite is also a polysaccharide and its effectiveness varies according to the type of salts in the media. In this regard, when used in Gamborg's B5 medium with agar, the rooting stage which has played an increasing role in the shoot growth during growth. Concerning the number of shoots, it was observed that a large number of sibling explants was quite sufficient for intensive and clonal production.

Table 8. The effect of the interaction between the "basal media x solidifier" of shoot numbers in rooting stage of *H. germanicopolitanum* explants (P≤0.05).

	Solidifier	
Basal Media	**Agar**	**Gelrite**
MS	11.296(cd)*	6.950(e)
Gamborg's B5	**29.842(a)**	11.696(cd)
N&N	15.993(b)	10.224(de)

*Different letters indicate significant differences between all the treatments.

Acclimatization

Concerning shoot and root growth, 100 plants representing the *H. germanicopolitanum* plant were taken to the acclimatization. The plants were planted in outdoor runs after the growth chamber and greenhouse stage. Thirty-two of these plants adapted to external conditions (Figure 3h-i). In the *in vitro* micropropagation study of *H. marminorense*, explants that provided shoot regeneration managed to acclimate to 97% external conditions [25]. Hamza and coauthors (2013) acclimated *H. lippii* L. var *sessiliforuim* samples to external conditions by 90-95%, and after two months, they fully acclimated 60% of the samples [27]. Morte & Honrubia (1992) achieved 95% success in their acclimatization studies in *H. almeriense* Paul [31]. Also, Hamza & Neffati (2014) were 90% successful in accustoming *H. kahiricum* Dell. to external conditions [28]. Due to the

fact that *H. germanicopolitanum* is local endemic and highly sensitive for in vitro micropropagation, its success in the acclimatization stage was lower compared to other studies.

CONCLUSION

Nowadays, people demand medicinal, aromatic plants in addition to chemical drugs. Thanks to the bioactive products and secondary metabolites contained in *Helianthemum* sp., its traditional use in the treatment of many diseases has made it an important position among medicinal and aromatic plants. To our knowledge, in this study, in vitro propagation of *H. germanicopolitanum* Bornm. was conducted for the first time in the literature. In vitro propagation is one of the most important methods of conservation of germplasm and their rapid and intensive reproduction. This is a very comprehensive optimization study in terms of testing three different media, 64 different PGR combinations, and two different solidifiers used in the initial stage. For *H.germanicopolitanum*, the success achieved at the shoot multiplication rate (94%), rooting rate (92%) and the acclimatization stage (32%) is quite high and can be considered as successful compared to other studies. Our findings can be used as an important preliminary resource in research on the *in vitro* propagation and regeneration of *H. germanicopolitanum* and for other endemic plants, which will be studied in the future.

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