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Germination and *in vitro* multiplication of *Helianthemum kahiricum,* a threatened plant in Tunisia arid areas

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Seeds of *Helianthemum kahiricum* have an excellent germination rate extending to about 90% within short time not more than four days after scarification (mechanical treatment) and a good protocol of disinfection. A high frequency of sprouting and shoot differentiation was observed in the primary cultures of nodal explants of *H. kahiricum* on Murashige and Skoog medium (MS) free growth regulators or with less concentration of kinetin (0.5 mg L^{-1} or 1.0 mg L^{-1} kin). *In vitro* proliferated shoot were multiplied rapidly by culture of shoot tips on MS with kinetin (0.5 mg L^{-1}) which produced the greatest multiple shoot formation. The kinetin had a positive effect on the multiplication and growth, but a concentration that exceeded 2.0 mg.L^{-1} decreased the growth. A high frequency of rooting with development of healthy roots was observed from shoots cultured on MS/8 medium hormone-free.

Key words: *Helianthemum kahiricum*, *in vitro* germination, multiplication, axillary buds.

INTRODUCTION

Helianthemum kahiricum (called R'guiga or Chaal in Tunisia) (*H. kahiricum*) is a perennial herb widely found around the Mediterranean basin (Raynaud, 1987). *H. kahiricum* is an appressed, grey-canescent perennial low shrub that reaches up to 10-25 cm long with many, branched stems. Leaves are 0.5-1.8 x 0.15-0.3 cm, oblong-lanceolate, appressed-pubescent, with strongly revolute margins, and acute to obtuse apex. Flowers are with white-villous, violaceous calyx and yellow petals equaling the sepals and are often not opening and arranged in 5-l2-flowered and 1-sided inflorescence. The fruit is an ovoid-globose and hairy capsule with ovoid-compressed, smooth, and brownish. This is a chaméphyte (Escudero et al., 2007), family of Cistaceae, arid regions and semi-arid areas (Perez- Garcia and Gonzalez- Benito, 2006). Despite its ecological and economic interests, this plant is a rare endemic flora of the western basin of the Mediterranean (Escudero et al., 2007), as a result of overgrazing (Aidoud et al., 2006). The size of their population or their range, or both, is

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Abbreviations: Zea, Zeatin (cytokinine); **IAA,** indole-3-acetic acid (auxin); **IBA,** indole-3-butyric acid (auxin); **Kin,** kinetin (cytokinine); **MS,** Murashige and Skoog medium; **NAA,** 2-naphthalene acetic acid (auxin); **2iP,** 2 isopentenyladenine (cytokinine).

Table 1. Culture medium for *Helianthemum kahiricum* seeds germination.

Concentration (mg L^{-1})	Culture medium				
	M_0	M_1	M_2	M_3	M_4
Kin	0	1	1	1	1
IBA	0	0	0.5	1	0.5
Zea	0	0	0	0	0.5

Table 2. Composition of culture medium with growth hormone (auxin and cytokinin) for the multiplication of *Helianthemum kahiricum* explants.

Concentration (mg L^{-1})	Culture medium						
	MS	C_1	C_2	C_3	C_4	C_5	C_6
Kin	0	0.5	0.5	0.5	1	2	2
IBA	0	0	1	0	0	1	0
NAA	0	0	0	1	0	0	1

restricted or is greatly diminished. The data indicates that the situation will worsen irreversibly if nothing is done to address this vulnerability. In other words, if the observed situation continues, it will anticipates the disappearance of these species more or less. Among the factors responsible, including loss or degradation of habitat, exploitation of the species, exposure to pollutants, diseases, climate change, overexploitation or other cause result in regression of range or a sustained decline in the number but the population level reaches a critical threshold. The helianthemes have a great pastoral interest. They have a very important role in the fight against desertification and stabilization of vulnerable areas (Diez et al., 2002). In addition, they are involved in the production of desert truffles (Al-Rahmah, 2001; Slama et al., 2006). Desert truffles, known locally as the "terfess" are edible and wild seasonal mushrooms hypogeal (Mandeel et al., 2007). *H. kahiricum* is considered threatened in an extremely precarious situation. The size of their population or their range, or both, is restricted or is greatly diminished. Thus, to maintain the genetic integrity of clones and conservation of this species, *in vitro* germination, cultivation microcuttings and stimulation of axillary buds are the most applied in plant micropropagation method. For the species used in this study, *in vitro* culture seems to be a very interesting alternative for preserving *H. kahiricum*. Therefore, the purpose of this work was the multiplication of the species by micropropagation highlighting the effect of the composition of the culture medium on the initiation and proliferation of plant.

MATERIALS AND METHODS

Plant material

The plant material used consists of *H. kahiricum* seed from region of Médenine (Benguerdenne: latitude 32°57'09"N, longitude 11°38'26"E, with an arid climate of an average rainfall of 150 mm/year and a sandy soil (Le Floch and Boulos, 2008). The explants which are internodes were taken from 2 months aged mother plants produced *in vitro*.

In vitro germination

Three experiments were conducted by changing the time and the type of fungicide to optimize better the germination of *H. kahiricum* seeds on MS medium (Murashige and Skoog, 1962). In all experiments, 20 seeds/replication were soaked in sodium hypochlorite followed by rinsing with distilled water. The seeds were disinfected in 70° alcohol followed by rinsing with sterile distilled water and then applying different fungicides: 1st experiment (Exp. 1): The seeds were treated with benlate (1 g L^{-1}) (10 min), followed by rinsing with distilled water; 2nd experiment (Exp. 2): The seeds were treated with mercuric chloride (1 g L^{-1}) (20 min), followed by rinsing with distilled water; 3nd experiment (Exp. 3): Without fungicide.

After the development of an adequate protocol of seeds disinfection, they were germinated in vials (20 seeds/vial). The germination test was carried out on MS (M_0) and MS modified with growth hormones (M1, M2, M3 and M4) (25 repetitions for each test) (Table 1). Incubation takes place in a growth chamber under controlled conditions with an alternating day/night (16:8 h), a relative humidity of 80 and 45%, respectively, and a temperature of 25 ± 2°C for one week.

In vitro multiplication

The vitropousses from *in vitro* germination were then stripped of their leaves, cut in microcuttings (1 or 2 cm) with 1-2 nodes. Ten explants per vial were transferred to different Culture medium: MS, MS supplemented with growth hormones (C1, C2, C3, C4, C5 and C6) (25 rehearsals for each test), and MS diluted (MS/2, MS/4, MS/6 and MS/8) (25 rehearsals for each test), without hormones (Table 2), with a view follow the morphological attributes such as, bud, the length of the main stem, number of leaves, number of nodes, root length and rooting rate. After their rooting, the vitropousses were transferred into plastic pot filled with sand to

ensure their adaptation and possible culture in an experimental plot for acclimation.

Statistical analysis

The results of analysis of variance (ANOVA) of the different parameters were obtained by the software SPSS v.11.5. Multiple comparisons of means and the setting command classes were made by Duncan's test.

RESULTS

In vitro germination

The success of *in vitro* culture depends on aseptic conditions for the cultivation of plant material used. After incubation, the best germination rate and reduced number of seeds contaminated are generated by the first test disinfection (100%) (Exp. 1). The second disinfection test (Exp. 2) yielded an average percentage of germinated seeds (32%) while the third disinfection protocol yielded no germination (0%) (Exp. 3). Seeds of *H. kahiricum* have an excellent germination rate; can reach 100% within short time not more than four days after scarification (mechanical treatment) and disinfection (Benlate). Scarification reduces germination time; it can stretch to several months because of the very rigid tegument of seeds.

Effect of growth hormone on seed germination

The seeds of *H. kahiricum* germinated on culture medium supplemented with growth hormone have present deformation (after germination) which results in vitrification and intense hydration with the formation of callus and necrotic leaves (Figure 1b, c, d, e and f).

Growth hormones have adverse effects on seed germination and thereafter are not effective for the germination of *H. kahiricum* seeds; while, the seeds on MS without growth hormones present a simple germination (Figure 2a).

Effect of growth hormone on micropropagation of *H. kahiricum*

Multiplication on MS medium without growth hormone

Shoots on MS medium are vigorous with chlorophyll leaves, normal and average percentage of vitrification. Morphological changes of vitropousses during subcultures affected neither leaves nor the appearance of the root. The leaves of plantlets contained the chlorophyll pigments, not necrotic, their size increases a subculture to another and becoming stronger. The stems reached a remarkable stiffness and strength during subcultures (Figure 1g, h and i). In *H. kahiricum*, it seems that the rooting phase is characterized by the proliferation of shoots on MS without growth hormones. Indeed, the time for obtaining vigorous shoots able to be transplanted is 8 to 12 weeks and it was during this period that shoots rooted (Figure 1j). Thereafter, the rooted vitropousses from the multiplication phase was prepared for the acclimatization stage.

Multiplication on MS medium supplemented with growth hormones

Recovery and proliferation axillary

Axillary buds subcultured on culture medium contains combinational hormones after six weeks of culturing explants bud unlike in the control medium, it takes place after two weeks (Figure 2a). MS has the highest rate of bud break (almost 90%). The effect of growth hormones is manifested by a significant reduction in the bud. The addition of the NAA causes an increase in the rate of bud compared to IBA. This can be explained by the physiological response of the explants on the interaction auxin/cytokinin, and the nature of the substance used for growth. The average shoot length appears to be strongly related to the concentration of hormones (Figure 2b). Using the results of the percentage of bud, it appears that the hormonal combinations recording bud rate reduced compared to the control. The influence of increasing the concentration of kinetin in the medium alone is shown by a significant decrease in the average length of the shoots.

Average number of nodes

The combination of auxin and cytokinin in the culture medium indicates that the increase in the kinetin medium, alone or in combination with auxin results is in a decrease in the average number of nodes relative to the control (Figure 2c). At a concentration of 0.5 mg L^{-1} Kin (C1), the average number of nodes reduced to about 20% whereas for 1.0 mg L^{-1} Kin (C4) it was in the order of 25%. In addition, 0.5 mg L^{-1} kin combined with 1.0 mg L^{-1} NAA (C3) gave a greater than 50% reduction compared to the control and the combination C2 (0.5mg L^{-1} kin + 1.0 mg L^{-1} IBA). It seems that the addition of growth regulators had a regressive effect on the average number of nodes.

Rooting

The cuttings rooted after three weeks in MS, then after a month we observed the emergence of some very harsh roots of short length on media supplemented with growth regulators. It appears that the observed formation of

Figure 1. (a) Setting seed germination of *H. kahiricum*. **(b)** Seeds of *H. kahiricum* germinated on MS. **(c)** *H. kahiricum* seeds germinated on MS + 1 mg/l Kin + 0.5 mg/l IBA. **(d)** *H. kahiricum* seeds germinated on MS 1 mg/l Kin + 1 mg/l IBA. **(e)** *H. kahiricum* seeds germinated on MS + 1 mg/l Kin. **(f)** Seeds germinated on MS + 1 mg/l Kin + 0.5 mg/l IBA + 0.5 mg/l Zea. **(g)** Appearance of leaves during 4th subculture. **(h)** Appearance of the stems during the 4th subculture. (i) Appearance roots for 3th subculture. (j) *In vitro* plants rooting.

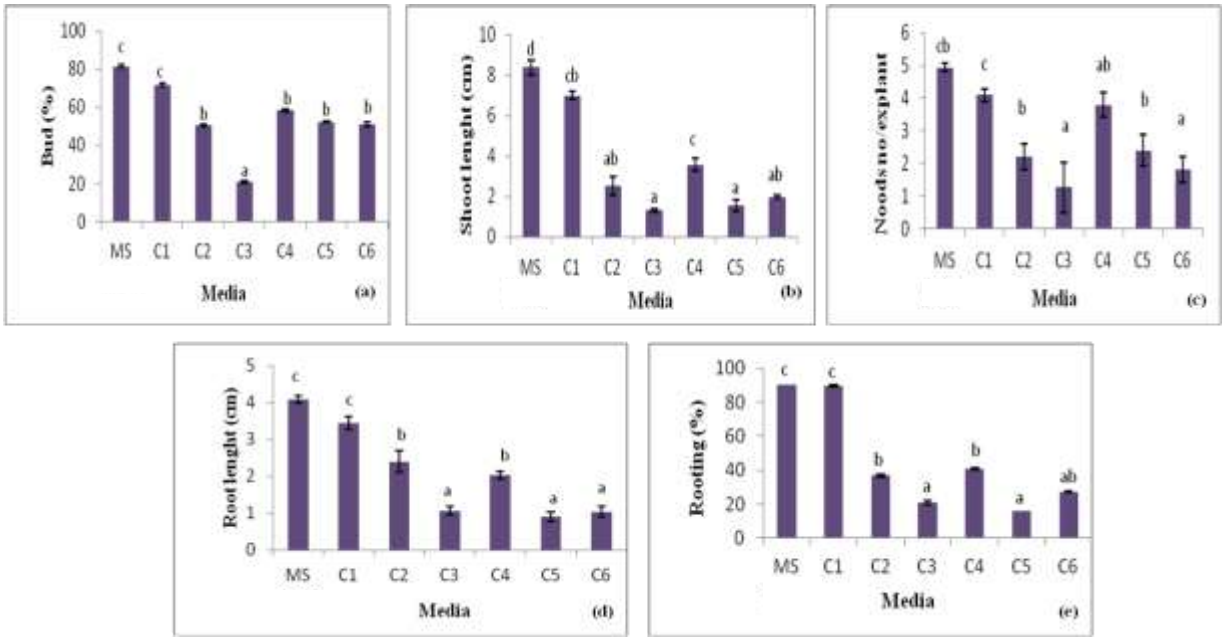

Figure 2. (a) Action of culture medium on the percentage of axillary buds of *H. kahiricum*, (b) action of media on the average length of *H. kahiricum* shoots, (c) action of media on average number of nodes, (d) effect of media on the average root length (cm) of *H. kahiricum* explants, (e) effect of media on rooting rate of *H. kahiricum* plantlets.

Table 3. Change in callus, bud break and rooting with hormone combinations of auxin/cytokinin in seedlings of two months old *Helianthemum kahiricum* from explants.

Explant	Culture medium				
	MS	C_2	C_3	C_5	C_6
Callus (%)	16±0[a]	78.4±0.78[b]	87.6±0.73[b]	91.37±0.59[c]	71.1±0.74[b]
Bud (%)	81.4±0.78[c]	50.62±1.00[b]	20.8±1.33[a]	52.4±0.88[b]	51.0±0.73[b]
Rooting (%)	90.4±0.48[d]	36.8±0.59[c]	21.1±0.71[b]	16.0±0.39[a]	27.4±0.48[ab]

Means with different letters are significantly different at threshold p<0.05 (Duncan test).

Table 4. Effect of MS dilute of the attributes morphological seedling of *Helianthemum kahiricum* aged two months from explants.

Explant	Culture medium			
	MS/2	MS/4	MS/6	MS/8
Buds (%)	90	100	100	100
Shoots length (cm)	2.24[a]	4.3[b]	5.7[c]	8.02[d]
Roots length (cm)	9.2[a]	12.4[b]	14.4[c]	15.3[d]
Leaves no/shoot	4.2[a]	6.2[b]	7.2[c]	7.7[c]
Nodes no/shoot	7[b]	6.3[a]	7.4[ab]	12.1[c]
Roots no/shoot	2.36[a]	2.44[a]	2.4[a]	3.01[b]

Means with different letters are significantly different at threshold p<0.05 (Duncan test).

callus at the base of the explants and at the location of sections inhibited rooting. Cuttings made on media containing no auxin (C1 and C4) showed highest rooting percentage. It also turns out that the addition of auxin in the medium does not improve rooting. Whatever the amount of kin in the medium, we find that IBA promoted rooting with a rate of 16% and the NAA with a rate of 21%. The rooting percentage and mean root lengths evolved in a proportional manner (Figure 2e).

Effect of ratio auxin/cytokinin on explants of *H. kahiricum*

The auxin/cytokinin ratio determines the physiological functioning of the explants, the percentage of callus is quite high compared to the control for all hormonal combinations (Table 3). However, the rate of rooting and sprouting of control are significantly higher compared to other treatments. The ratios of auxin/cytokinin about 2 (1:0.5 mg L^{-1}) promote rooting, indeed, for the combination C2 (1:0.5 mg L^{-1}) rooting percentage was 41% against a rate of 16% for the combination C5 (1:2 mg L^{-1}). The C5 (1:0.5 mg L^{-1}) promote axillary bud (62%), while C3 (1:0.5 mg L^{-1}) promoted it by 22%. However, the dilution does not influence the rate of bud, but it manifests itself on the strength of axillary shoots. There is also a strong ability of budding *in vitro H. kahiricum* associated with its proliferation of axillary explants in the presence of all medium (Table 4).

Acclimatization

The survival rate of plantlets is influenced by the nature of the substrate. The best survival rate of seedlings (63%) was recorded on a substrate perlite.

DISCUSSION

H. kahiricum is considered threatened in an extremely precarious situation. Micropropagation of this plant was an attempt conducted from nodal explants of plantlets *in vitro* to preserve/conserve this species by the micro-propagation technique which aims to safeguard bio-diversity; thereby developing an appropriate protocol for disinfecting seeds which was necessary to achieve aseptic cultures. Seed germination after strong disin-fection has the minimal contamination and a high rate of seeds germinated on MS medium without hormone. It appears that *H. kahiricum* does not require growth hormone for its germination. Hence, the study was based on regeneration by *in vitro* proliferation of axillary buds of *H. kahiricum* by the use of growth hormones in order to improve the multiplication. Microcuttings grown on MS medium without hormone presents the highest rate of budding and rooting. It seems that this plant has high potential to multiply on a hormone-free MS medium and the bud of the axillary shoots is easy without going through an induction medium. Growth substances (auxins and cytokinins)

have a depressive effect on budding, proliferation and rooting of plantlets. The acclimatization of *H. kahiricum* poses no major problems and the use of substrate consisting of sand and perlite has a high survival rate. It seems that the buds during the multiplication are actually inhibited by the effect of callus at the base of *H. kahiricum* explants. Therefore, it seems that the hormonal combination has a regressive effect on bud break, rooting and average root length; in addition, these parameters appear to be influenced by the nature of the auxin. The addition of growth hormones does not improve the rooting or the rooting period compared to the control environment. Similar results were also reported by Souayah et al. (2003) in *A. halimus* for which the root is obtained on media without growth regulators with a rooting rate improved by diluting the mineral medium; while, the work of Roy et al. (2001) on *Humulus lupulus* and Armstrong and Johnson (2001) on *Ceratopetalum gummiferum* showed that in the absence of any growth regulator, rooting could not be obtained. This may be due to the richness of this species in endogenous growth regulators. Fracaro and Echeverrigaray (2001) reported that for *Cunila galioides*, dilution of the mineral medium does not affect the induction of rooting, but negatively affects the growth of roots. However, *Pistachia vera* (Chatibi, 1999), dilution of macronutrients MS basal medium bears no significant improvement for rooting and for absence of auxin, no rooting was observed.

Conflict of interests

The authors did not declare any conflict of interest.

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