J. Agric. Sci. Mansoura Univ., 25 (10): 6397 - 6408, 2000.

COMPARATIVE STUDIES ON *Hypericum perforatum* L. *IN VIVO* AND *IN VITRO*

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ABSTRACT

The effect of normal and continuous day light (White lamps 3000 Lux) as well as, cold treated or untreated seeds on propagation of *Hypericum perforatum* L. *In vivo* and *In vitro* were studied. It was found that: *In vivo*, the cold treated seeds with 4°C for 30 days before cultivation under continuous light showed the best results, i.e. higher germination percentage; plant height (cm); greater number of branches/plant; fresh and dry weight (gm/plant) and dry matter content (%) as compared with untreated seeds which cultured either under normal or continuous day light. In vitro studies, the obtained plantlets from cultivation of cold treated seeds on MS-agar medium supplemented with 0.05 mg/l NAA + 0.5 mg/l BA, and incubated at 26 ± 1°C for three months then, transferred into greenhouse under continuous day light, showed the best results on obvious determinations than untreated seeds or treated seeds and cultured under normal day light. Propagation of *Hypericum perforatum* L *In Vitro* showed better results than *In Vivo*.

INTRODUCTION

Hypericum perforatum L.,plant Fam. Hypericaceae grown naturally in Europe, Far East, Werstern Siberia, North West China, Asia and North Africa (Campbell and Delfosse. 1984). The aerial parts are used for medicine, containing pharmaceutical components i.e., hypericin and pseudohypericin (Cellarova *et al*., 1996). It affects on healing wounds and antidepressive properties (Zdunk and Alfermann,1992). Also, in folks medicine it has been used as herbal medicine, i.e. antimalarial (Bombardelli and Morazzoni, 1995). The extracts are well know as antibiotics, used as antiviral agents, beneficial against sores and burns as an infusion, also, they act against pulmonary and urinary infection (Towers,1980; Crompton *et al*., 1988).

Recently it had been found that hypericin and pseudohypericin are effective against certain retro-viruses (Meruelo *et al.*, 1988; Lavie *et al*., 1990 and Degar *et al.,* 1992). This plant grows well in Europe, while in Egypt it is unknown. However it is advisable to introduce such plant into Egypt to be studied and may be become one of the exportable medicinal plants to cover part of foreign market or to be introduced for local industries of medicines. The demand is increasing from one year to other. Thus, this study had been conducted to study its propagation from seeds treated with low temperature (4°C) for 30 days or untreated seeds before cultivation under different light conditions.

MATERIALS AND METHODS

The experiments of this study were carried out in the Laboratory of Tissue Culture and greenhouse at the Department of Ornamental Horticulture, Faculty of Agriculture, Cairo University, Giza, during 1997. The aim of this investigation was to study the effect of cold treatment of seeds at 4 °C for 30 days before its cultivation *in vivo* and *in vitro* , under different light conditions, i.e., normal day light and continuos artificial cool white light in comparison with the untreated seeds. Seeds of *Hypericum perforatum* L. were obtained for the first time in Egypt from Prof.Dr. Hölzl Institute of Pharmaceutical Biology, Philipps Univ., Marburg, Germany.

The obtained seeds of Hypericum were divided as follows:

1-In vivo studies:

a.Portion of the untreated seeds were cultivated directly in a mixture of Peatmoss + Sand (1:1) under greenhouse condition with 16 hrs. day light, while other portion was cultured at continuos light condition (3000 Lux from cool white fluorescent lamps).

b.Treated seeds of *Hypericum perforatum* L.with 4 °C for 30 days before cultivation were cultured in the mixture of Peatmoss + Sand (1:1) in the greenhouse under the prementioned conditionss of lightening.

The treatments were repeated three times with ten replicates:

2-In vitro studies:

Both of the treated or untreated seeds of *Hypericum perforatum* L, were cultured after sterilization by washing with water, followed by rinsing in Ethanol 70% for 30 Seconds and then immersed in Clorox 50% for 20 minutes including drop of Tween 20. After that, seeds were washed several times with redistilled sterilized water.

The cultivation occurred on prepared solid MS-medium either free hormones or that supplemented with 0. 05 mg/l NAA + 0.5 mg/l BA (These concentrations were obtained from preliminary studies on the favorable concentrations of direct regeneration plantlets). The pH of all cultures were adjusted at 26 ± 1°C in growth chamber for three months. Plantlets were transferred into mixture of peatmoss + Sand (1:1) and maintained under normal day light or continuos lighting conditions (3000 Lux for cool white florescent lamps).

The recorded data were: percentage of germination (weekly), plant height, number of branches/plant and percentage of dry matter content (monthly)

Statistical analysis

All experiments were designed in a completely randomized design and obtained data were statistically analyzed using standars error (SE) according to the method described by Snedecor and Cochran (1967).

RESULTS AND DISCUSSIONS

Germination percentage :

Germination of the cold treated seeds at 4°C for 30 days before cultivation was higher for that cultivated under continuos light as compared with that cultivated under normal day light giving 97.3 and 99.7 % *In vivo* and *In vitro*, respectively.[Table 1 and Fig.1].

Fig.(1): Germination of *Hypericum, perforatum* L. seeds cultured directly on MS- medium as follow:
1- Free hormones
2- Supplemented with 0.05 mg/l NAA + 0.5 mg/l BA.

In case of the untreated seeds, the germination percentage increased to reach its maximum in the 4 th week *in vivo* giving 91 $\pm$ 0.58 and 93 $\pm$ 0.90 for normal day light and continuous light , respectively. This means that treating seeds of *Hypericum perforatum* L. with 4°C for 30 days and cultivation under continuous light was favourable for increasing the germination percentage of seeds by enhancing its embryo to germinate better than the untreated seeds.

In vivo the height values for plantlets produced from untreated seeds increased from (4.6 cm) in the first month to reach (65.0 cm) at the age of 6 months. This occurred under day light condition, while taller plants were measured under continuous light, which gave 6.1 cm at the first month then increased to reach 18.1, 42.8, 59.5, 62.0 and 72.8 cm for the 2 nd 3 rd 4 th 5 th and 6 th month, respectively. This means that, continuous light was more effective on increasing the plant height as a result of its effect on stimulation of cell elongation.

Fig.(2):Plant height of *Hypericum perforatum* L plant as affected by cold temperature treatment and light conditions:

A: *In vivo* **1- Untreated seeds under normal day light.**
2- Untreated seeds under continuous day light.
3- Treated seeds with 4 °C under normal day light.
4- Treated seeds with 4 °C under continuous day light

B: *In vitro* **1- Untreated seeds under normal day light.**
2- Untreated seeds under continuous day light.
3- Treated seeds with 4 °C under normal day light.
4- Treated seeds with 4 °C under continuous day light

In case of the treated seeds, the values of plant height under *in vivo* culturing were higher under both conditions of illumination, i.e. normal day light or continuous light. The recorded values after 6 months of cultivation were 77.7 and 86.6 cm for the two light conditions, respectively.

In vitro, the untreated seeds resulted in 35.5 and 38.0 cm after 6 months of cultivation on MS- medium containing hormones (0.5 mg/l BA + 0.05 mg/l NAA), whereas the values were taller for that cultivated on MS free of hormones which gave 44.0 and 50.3 cm, for day light or continuous light conditions, respectively.

Treating seeds with low temperature enhanced plant height to reach 41.9 and 43.2 cm as well as 56.0 and 60.3 cm for cultures under day light or continuous light conditions, cultivated on MS-medium supplemented with hormones, respectively. This may be due to the presence of exogenous hormones such as auxin (NAA) and cytokinin (BA), which stimulated cells endogenous hormones and subsequently enhance cell division and cell elongation.

Number of branches / plant:

Data in Table 3 and Fig.3 revealed that, the number of branches/ plant has been affected with cultivation of untreated or treated seeds with that concluded by Ammarito, 1983, who found that, auxin and cytokinin encourage the multiplication of cells and branching of plants.

Fresh and dry weight (gm/plant):

The data in Table (4) showed fresh and dry weight /plant of Hypericum *in vivo* or *in vitro* cultivation. In case of *in vivo* cultivation, fresh and dry weight were increased gradually as the age of the culture increased. The values for either untreated or treated seeds with cold temperature (4°C) were higher, when cultivation was done under continuos light as compared with that under day light. Moreover, the treated seeds produced heavier plants than untreated seeds.

In case of *In vitro* cultivation, the obtained values were higher for the culture containing hormones, than that free of hormones. The values for the treated seeds were higher than the untreated ones, the highest values for the treated seeds and cultivated under continuos light by using MS-medium supplemented with hormones. This means that, cold treatment was favorable for plant growth and accumulation of dry matter under enough light (continuous light). The presence of BA and NAA were helpful for cell division.

Fig.(3):Number of branches of *Hypericum perforatum* L. plant as affected by treatment of cold temperature (4 °C) and cultured under different light conditions:

1- Untreated seeds under normal day light.
2- Untreated seeds under continuous day light.
3- Treated seeds with 4 °C under normal day light.
4- Treated seeds with 4 °C under continuous day light

Dry matter percentage:

Data in Table (5) showed that, the percentage of dry matter was increased as the plant age was increased . The treated seeds produced higher percentage as compared with the untreated seeds, either for that *In vivo* or *In vitro* cultivation. Moreover, in case of *in vitro*; the addition of BA and NAA affected positively on the percentage of dry matter. The cultivation under continuous light was more effective on increasing dry matter percentage, than cultivation under day light, i.e. more lightening was effective as assimilation and formation of stored material.

In case of the effect of continuous light condition on vegetative and flowering growth pattern it was found that, the obtained results are in close with that found by Banko and Stefani 1991 who concluded that vegetative growth and most flower bud of *Oxydendrum arboreum* explants were developed with a 24 hr. photoperiod.

On the other hand, the effect of low temperature on development of embryos seeds were investigated by Coumans- Gilles *et al*., 1981 in *In Vitro* culture of sugar beet. They reported that, seeds of sugar beet seeds requre 4 °C for 30 days before cultivation to complete germination as well as good multiplication.

Table (5):Dry matter content (%)of *Hypericum perforatum* L plants induced from treated and untreated seeds cultured *in vivo* or *in vitro* and incubated under normal and long day light , during 6 months from cultivation.

Treatments / Months	*In vivo*				*In vitro**							
	Untreated seeds		Treated seeds (4 °C)		Untreated seeds				Treated seeds (4 °C)			
					A		B		A		B	
	1	2	1	2	1	2	1	2	1	2	1	2
1 st Month	1.18	1.49	1.66	1.87	---	---	---	---	---	---	---	---
2 nd Month	1.58	2.2	2.41	2.81	---	---	---	---	---	---	---	---
3 rd Month	3.38	4.23	4.58	5.3	---	---	---	---	---	---	---	---
4 th Month	4.18	5.3	5.71	6.2	5.03	5.5	3.34	3.63	5.65	6.26	5.39	5.9
5 th Month	5.59	6.2	6.8	7.44	7.0	7.5	4.0	5.68	7.77	7.83	7.29	7.74
6 th Month	6.07	7.24	8.29	8.64	7.99	8.49	7.12	8.28	8.95	9.28	8.44	8.55

= 1,2,3 months cultured *In vitro* 4,5,6 months cultured *In vivo.
± = SE Standard error
1 = Normal day light 2 = Long day light.
***In vivo* = Peat moss + Sand (1:1) in greenhouse.**
***In vitro* = MS-medium supplemented with:**
A = With hormone (0.5 mg/l BA + 0.05 mg/l NAA).
B = Without hormone = Basal MS-medium.

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دراسات مقارنه على اكثار نباتات الهيبريوكم معمليا و غير معمليا

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اولا: الاكثار الغير معملى : من النتائج المتحصل عليها ثبت ان معالجه بذور نبات الهيبريوكم بمعدل ٤ °م (٣٠ يوم) قبل زراعتها فى الصوبه الزجاجيه تحت ضروف الاضاءه المستمره من لمبات فللورسنت (ضوء ابيض ٣٠٠٠ لوكس) اعطت احسن النتائج فى نسبه الانبات (%) , ارتفاع النبات (سم) , عدد الافرع المتكونه لكل نبات , الوزن الطازج و الجاف (جرام / نبات) و كذلك النسبه المئويه للرطوبه عن البذور المعامله و التى زرعت تحت ظروف الاضاءه العاديه و كذلك عن الغير معامله و التى زرعت تحت ظروف الاضاءه العاديه او الاضاءه المستمره.

ثانيا: الاكثار المعملى : النباتات المتحصل عليها معمليا من زراعه البذور المعامله على ٤°م (٣٠ يوم) قبل زراعتها على البيئات الصلبه (موراشيج-سكوج) المضاف اليها ٥., ملليجرام/ لتر نفثالين حمض الخليك + ٥, ملليجرام/ لتر بنزيل ادينين ثم زرعت فى الصوبه تحت ظروف الاضاءه المستمره اعطت احسن النتائج عن الغير معامله او المعامله و المنزرعه تحت ظروف النهار العادى او تحت ظروف الاضاءه المستمره. مما سبق يمكن التوصيه باكثار نباتات الهيبريوكم معمليا عن طريق معالجه بذور نبات الهيبريوكم بمعدل ٤ °م (٣٠ يوم) ثم زراعتها على البيئات الصلبه (موراشيج-سكوج) المضاف اليها ٥., ملليجرام/ لتر نفثالين حمض الخليك + ٥, ملليجرام/ لتر بنزيل ادينين ثم زراعتها فى الصوبه تحت ظروف الاضاءه المستمره.

Table (1): Germination percentage for seeds of *Hypericum perforatum* L., as affected by cold storage (4 °C for 30 days) before culturing *in vivo* and *in vitro*.

Treatments / Weeks	*In vivo*				*In vitro*							
	Untreated seeds		Treated seeds (4 °C)		Untreated seeds				Treated seeds (4 °C)			
					A		B		A		B	
	1	2	1	2	1	2	1	2	1	2	1	2
1 st Week	35.7±2.7	41±2.64	46.7±2.0	52.3±1.8	23±2.08	30.7±1.3	55.6±2.8	58.2±0.6	32.7±1.5	40.6±0.8	60.8±0.2	62.4±1.5
2 nd Week	74±3.78	81.3±1.9	88±1.73	92.3±1.2	60.3±5.5	67.6±0.7	89.9±3.4	91.8±1.5	67±4.5	78.2±1.6	92.5±0.6	96.4±2.6
3 rd Week	88.7±0.9	89±0.6	92±1.15	94.7±0.9	84±1.15	90.6±2.6	95.3±1.6	96.4±0.9	90.6±0.9	91±0.3	95.8±1.0	97.3±3.8
4 th Week	91±0.58	93.3±0.9	96±0.57	97.3±0.9	69.3±0.8	97±1.7	97.6±0.5	98.4±2.7	98±0.42	99.1±0.4	99.4±0.1	99.7±0.1

± = SE (Standard error)
1 = Normal day light
2 = Long day light.
***In vivo* = Peat moss + Sand (1:1) in greenhouse.**
***In vitro* = MS-medium supplemented with:**
A = With hormone (0.5 mg/l BA + 0.05 mg/l NAA).
B = Without hormone = Basal MS-medium.

J. Agric. Sci. Mansoura Univ., 25 (10): 6397 - 6408, 2000.

Table (2): Plant height of Hypericum perforatum L (cm) plant as affected by treating seeds with low temperature (4 C) and different light conditions cultured *In vivo* and *In vitro*.

Treatments / Months	*In vivo*				*In vitro**							
	Untreated seeds		Treated seeds (4 °C)		Untreated seeds				Treated seeds (4 °C)			
					A		B		A		B	
	1	2	1	2	1	2	1	2	1	2	1	2
1 st Month	4.6±0.6	6.1±0.2	6.3±0.3	14.3±2.3	0.7±0.12	1.0±0.1	2.3±0.4	3.6±0.4	1.3±0.5	1.5±0.12	4.6±0.8	4.9±0.6
2 nd Month	13.5±1.5	18.1±1.9	22.5±2.1	26.9±2.5	2.9±0.3	3.3±0.2	4.9±0.6	5.6±0.3	4.0±1.4	4.5±1.6	7.4±1.5	8.5±1.8
3 rd Month	39.0±2.5	42.8±3.5	46.8±3.4	60.2± 3.8	6.6±0.3	7.0±1.2	12.7±1.9	15.9±2.8	10.9±3.0	12.3±2.6	16.6±3.4	18.6±4.3
4 th Month	55.0±4.8	59.5±5.4	63.9±5.6	68.7±4.9	10.7±1.2	14.6±2.6	20.7±2.9	27.6±3.2	19.3±4.5	20.5±3.9	31.5±4.2	35.1±4.9
5 th Month	58.7±5.2	62.0±4.8	68.8±5.2	72.9±4.5	24.4±2.6	28.5±3.6	32.8±3.1	41.1±3.4	30.5±3.4	32.0±3.8	45.6±4.8	48.6±5.4
6 th Month	65.0±2.4	72.8±3.5	77.7±4.2	86.6±4.8	35.5±3.4	38.0±4.2	44.0±3.4	50.3±4.2	41.9±4.5	43.2±3.6	56.0±4.5	60.3±5.2

= 1,2,3 months cultured *In vitro* 4,5,6 months cultured *In vivo.
± = SE Standard error
1 = Normal day light 2 = Long day light.
***In vivo* = Peat moss + Sand (1:1) in greenhouse.**
***In vitro* = MS-medium supplemented with:**
A = With hormone (0.5 mg/l BA + 0.05 mg/l NAA).
B = Without hormone = Basal MS-medium.

Table (3): Number of branches/plant of *Hypericum perforatum* L induced from treated and untreated seeds cultured in vivo or in vitro and incubated under normal and long day light , during 6 months from cultivation.

Treatments / Months	*In vivo*				*In vitro**							
	Untreated seeds		Treated seeds (4 °C)		Untreated seeds				Treated seeds (4 °C)			
					A		B		A		B	
	1	2	1	2	1	2	1	2	1	2	1	2
1 st Month	2.33±0.3	2.67±0.3	3.0±0.00	3.33±0.3	4.00±0.6	5.2±0.54	3.1±0.3	3.4±0.56	5.6±0.8	6.2±0.57	4.0±0.3	4.5±0.6
2 nd Month	2.33±0.3	4.67±0.3	4.33±.0.3	5.33±0.4	5.7±0.6	6.0±0.2	4.0±0.56	4.5±0.3	6.0±0.33	6.7±0.56	4.50±0.3	5.9±0.8
3 rd Month	4.67±0.6	6.0±0.57	6.67±0.4	7.2±0.58	7.5±0.3	7.9±0.4	6.8±0.4	7.4±0.6	8.3±0.66	8.9±0.88	7.0±0.52	7.8±0.3
4 th Month	5.0±0.4	7.33±0.3	7.67±0.5	8.0±0.67	8.2±0.4	9.2±0.6	7.90±0.5	8.1±0.55	9.5±0.35	10.0±0.3	8.2±0.6	8.6±0.8
5 th Month	6.0±0.58	8.0±0.58	8.67±0.3	9.67±0.5	9.9±0.8	10.8±0.3	8.5±0.33	9.8±0.6	11.0±0.4	12.3±0.4	10.4±0.3	11.3±0.5
6 th Month	6.67±0.3	8.67±0.3	9.33±0.8	10.0±0.5	10.4±0.7	11.7±0.5	9.5±0.4	10.2±0.4	12.5±0.6	14.2±0.5	11.0±0.3	12.1±0.4

= 1,2,3 months cultured *In vitro* 4,5,6 months cultured *In vivo.
± = SE Standard error
1 = Normal day light 2 = Long day light.
***In vivo* = Peat moss + Sand (1:1) in greenhouse.**
***In vitro* = MS-medium supplemented with:**
A = With hormone (0.5 mg/l BA + 0.05 mg/l NAA).
B = Without hormone = Basal MS-medium.

Table (4): Fresh and dry weight of *Hypericum perforatum* L (gm/ plant) induced from treated and untreated seeds , cultured *in vivo* and *in vitro* and incubated under normal or continuous day light, during 6 months from cultivation.

Treatments	*In vivo*				*In vitro**							
	Untreated seeds		Treated seeds (4 °C)		Untreated seeds				Treated seeds (4 °C)			
					A		B		A		B	
Months	1	2	1	2	1	2	1	2	1	2	1	2
Fresh weight												
1 st Month	0.44±0.07	0.71±0.08	0.59±0.01	0.87±0.03	---	---	---	---	---	---	---	---
2 nd Month	1.19±0.02	1.88±0.06	1.58±0.14	2.01±0.05	---	---	---	---	---	---	---	---
3 rd Month	3.06±0.05	3.82±0.03	3.54±1.73	4.04±0.04	---	---	---	---	---	---	---	---
4 th Month	4.41±0.2	4.87±0.15	4.72±0.16	5.15±0.09	3.82±0.08	4.25±0.05	5.29±0.15	5.64±0.06	4.05±0.03	4.77±0.18	5.19±0.03	5.95±0.04
5 th Month	5.48±0.11	6.61±0.19	5.87±0.12	7.12±0.08	4.71±0.15	5.93±0.04	5.66±0.02	7.13±0.06	5.19±0.06	6.42±0.08	6.29±0.14	6.67±0.23
6 th Month	6.75±0.06	7.88±0.06	7.05±0.05	8.12±0.07	5.37±0.13	7.05±0.06	7.27±0.06	8.18±0.02	5.89±0.06	7.39±0.10	7.17±0.02	7.78±0.04
Dry weight												
1 st Month	0.0052± 0.001	0.011± 0.001	0.01± 0.003	0.016± 0.0001	---	---	---	---	---	---	---	---
2 nd Month	0.19± 0.001	0.041± 0.004	0.038± 0.0042	0.057± 0.006	---	---	---	---	---	---	---	---
3 rd Month	0.10± 0.005	0.162± 0.004	0.162± 0.006	0.21± 0.002	---	---	---	---	---	---	---	---
4 th Month	0.185± 0.01	0.26± 0.013	0.27± 0.01	0.32± 0.005	0.27± 0.006	0.31± 0.003	0.13± 0.01	0.15± 0.002	0.29± 0.004	0.37± 0.01	0.22± 0.002	0.29± 0.02
5 th Month	0.25± 0.006	0.398± 0.012	0.40± 0.01	0.53± 0.007	0.40± 0.001	0.35± 0.005	0.19± 0.006	0.34± 0.002	0.50± 0.01	0.52± 0.006	0.37± 0.004	0.49± 0.004
6 th Month	0.423± 0.007	0.57± 0.016	0.59± 0.007	0.70± 0.02	0.58± 0.005	0.70± 0.002	0.38± 0.009	0.58± 0.003	0.64± 0.005	0.72± 0.004	0.50± 0.02	0.63± 0.007

***1,2,3 = months cultured *in vitro* 4,5,6 months cultured *in vivo*. ± = SE Standard error 1= Normal day light 2= Continuous day light**
***In vivo* = Peatmoss + sand (1:1) in Greenhouse. *In vitro* = MS-medium supplemented with :**
A= With hormone (0.05 mg/l NAA + 0.5 mg/l BA) B = Without hormones Basal MS-medium

J. Agric. Sci. Mansoura Univ., 25 (10): 6397 - 6408, 2000.