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Original Article

Identifying the explants and hormonal concentrations of 2, 4-D and BAP for genetic resources in Micro-Propagation of *Hypericum Perforatum* L.

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ABSTRACT

Due to its geographical distribution problem, tissue culture propagation can be used for genetic resource and commercialization of *Hypericum perforatum L.* known as St John's wort, regarding its active ingredients such as hypericin, which is anti-AIDS and antidepressant. In this research, the way of callus induction and genetic resources were carried out on *Hypericum perforatum L.* by four explants, including root, stem, leaf, and hypocotyl. The explants were cultured in Murashige and Skoog (MS) medium along with two growth regulators, namely 2, 4-D at four hormonal levels (0.1, 0.2, 0.3, 0.4 mg/L) and BAP (0.25, 0.5, 1, 2 mg/L) in 16 hormonal treatments based on completely randomized design with 4 replications and each replicate containing 5 explants. According to data (callus number and diameter, dry weight, relative frequency) in each Petri, the highest amount of callus and genetic resource was achieved with 0.5 mg/L BAP and 0.1 mg/L 2, 4-D in root explant, and the lowest amount was at 0.5 mg/L BAP and 0.2 mg/L 2, 4-D in stem explant. Generally, a high hormonal concentration of BAP resulted in a decreased number of callus, relative frequency, and callus diameter. Furthermore, a high hormonal concentration of 2, 4-D caused an increased number of callus, relative frequency, descending diameter, dry weight.

Keywords: Genetic Resource, Callus, Hypericum Perforatum L., BAP, 2, 4-D.

Introduction

Plant tissue culture is one of the most effective techniques in plants mass production and gene transfer. Researchers use plant tissue culture capacities to produce disease-free plants and herbal medicines. Developing mass propagation protocols for medicinal plants is the aim of these researchers. New and practical methods of cell culture and tissue culture in the eugenics and growing of medicinal plants provide the opportunity to further exploit the medicinal plants.

Both in terms of treatment and prevention of diseases, medicinal herbs are of particular value and importance in the health and well-being of communities. This part of the natural resources is as old as human beings and has been one of the most significant sources of human food and medicine for generations [1]. Hypericum *perforatum L.* is one of the valuable medicinal plants in the family Hypericaceae [2]. In Persian sources, it is known as perfoliate St. John's wort, amber, and Aaron's beard [3]. In English, it is known as St John's wort [4]. This perennial hardwood plant belongs to the Clusiaceae and Hypericoideae family [5].

This plant is used as an astringent, skin softener, and cough suppressant in traditional medicine [6]. It has been used to treat neurological disorders, pulmonary diseases [7], neurasthenia, and depression [8]. Today, it is mostly being used to provide antidepressant [9] and insomniac medicines [10]. Furthermore, this plant can be used as an antiseptic cream for

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wounds and burning [11], and muscle cramp [12]. Regarding their fabulous property, St John's Wort has been known as the king of the medicinal plants [13].

The methods of genetic resources are classified into two main categories, including traditional and in-vitro ones [14]. In the traditional method, plant growth and propagation occur in its natural environmental condition [15]. The major problem of the use of this method can be summarized as the following:

1. Fail to conserve the genetic stability in sexual propagation (seed production), especially in micro-propagation of cross-pollinated plants [16].
2. Plants exposure to harmful biotic and abiotic environmental factors, resulting in the destruction of germplasm [17]. Lack of viability and appropriate storage in recalcitrant tissues would cause the repeated propagation times [18] and high maintenance cost and the risks of two previous cases [19].

Several studies have been conducted on the tissue culture of *Hypericum Perforatum L*. A study on *Hypericum Perforatum L.* set out to examine the root explant for callus induction with hormonal treatments of BAP (0.7, 1.7, 2.7) + NAA (0.5, 1, 2.2) mg/L. The results indicated that the best hormonal treatment for callus induction and genetic resources was 1 mg/L BA with 0.7 mg/L NAA [20].

Another research on *Hypericum Perforatum L.* was carried out to investigate root explant for callus induction with hormonal treatments of BA (0.1, 1) + 2, 4-D (0.1, 1) mg/L. This study demonstrated that the highest efficiency was 1 mg/L BA and 0.1 mg/L 2, 4-D in the hormonal treatments for regeneration and genetic resource [21].

One study investigated the hypocotyl explant for regeneration with hormonal treatments of BA (0.45, 0.9, 1.35) mg/L, NAA (0.8, 0.0186, 0.0093), and BAP (0.5, 1, 5). The findings pointed out that the highest amount of callus induction was achieved with 0.9 mg/L BA and 0 mg NAA. Furthermore, the highest amount of hypocotyl was in MS medium and 0.1 mg KIN^{+2} IBA [22].

In another research, the root explant was used for callus induction with hormonal treatments of NAA (0.5, 1, 2.5) + BA (0.25, 1,2.5) + 2, 4-D (0.25, 0.5, 1). The investigation of rooting results showed that the highest amount of rooting in terms of number and length of the root was in the treatments containing 0.5 mg/L BA (on average 4.3 roots per cuttings, the mean of toots length was 2.3 cm) [23]. Given that no technology was able to restore big destroyed ecosystems, species, and genes [24] and regarding the severe effects and consequences of biodiversity loss in agricultural, industrial, and medicinal achievements [25], it is important to take genetic resources into consideration [24]. With regard to the high therapeutic properties of *Hypericum Perforatum L.* and using medicinal plants instead of chemical treatments for its possible harms and as well as the availability of a few studies on other hormonal treatments towards regeneration of this plant, the present research aimed at identify the explants and hormonal concentrations (BAP, 2, 4D) for genetic resources in micro-generation of *Hypericum Perforatum L.*. Accordingly, it made an important contribution to the existence weaknesses on tissue culture data and explants and hormonal characteristics, providing additional information for those researchers who work on the subjects of tissue culture and genetic resources in plants.

Material and Methods

Seed collection and plant sample

This experiment started to identify the explants and hormonal concentrations (BA, 2, 4-D) for genetic resources in micro-propagation of *Hypericum perforatum L.* at the laboratory of tissue culture and agricultural physiology of Payame Noor University of Mashhad, Iran in October 2017. The seed plant of *Hypericum perforatum L.* was obtained from the Faculty of Agriculture, Ferdowsi University of Mashhad, Iran, registering with 1918 code. Its seeds were immediately isolated and conserved in the paper pockets and at cold milieu [26].

Seed culture and production of sterilized explants

In order to provide sterilized seedlings of *Hypericum perforatum L.*, its seeds were sterilized by immersing in 1.5% sodium hypochlorite for 15 minutes, followed by three washes with sterile distilled water (it is one of the sterilization methods of seed germination) [27]. After seed sterilization, the seeds of *Hypericum perforatum L.* were placed in the glass jars containing culture medium and they began to germinate after approximately 7 days. The treatments of callus induction including 4 explants (root, stem, leaf, and hypocotyl) were investigated in the culture medium of Murashige and Skoog (MS) [28]. Moreover, two types of 2, 4-D growth regulators were examined in four levels (0.1, 0.2, 0.3, 0.4 mg/L) and BAP (0.25, 0.5, 1, 2 mg/L) in 16 hormonal treatments was investigated as factorial based on a completely randomized design with 4 replications and each replicate containing 5 explants and under the dark condition at the temperature of 25 ± 2.

After 25 days, the amount of callus growth, their number in each Petri, callus diameter, their fresh and dry weight were measured and the best hormonal treatment was identified. Data analysis was carried out in 17.3 Minitab, and the diagrams were drawn by Excel.

Results and Discussion

According to the variance analysis, the simple effect of explant type and different hormonal level were significant for all measured traits at $P<0.01$. Furthermore, the interaction effect of different concentration of 2, 4-D and BAP hormones were significant for the traits of dry weight and callus diameter at 1% probability level, which it was closely inconsistent with the results of Fabiane and Eliane (2000) who examined the traits of dry weight and callus diameter. The interaction effect of different concentrations of hormone in the explant was significant for all traits being examined at the probability level of $P<0.01$.

Table 1. Variance Analysis of the Effect of Explant Type and Different Levels of BAP and 2, 4-D Hormones on Callus Induction and Some Traits

		Mean Square			
Sources of change	Degree of freedom	Number of Callus	relative frequency of callus	Dry weight of callus (g)	Callus diameter (cm)
Explant	3	**97.353	**38941.243	**0.058	**10.766
BAP	3	**4.953	**1981.087	**0.003	**1.213
Explant* BAP	9	^{ns}0.829	^{ns}331.434	**0.003	**0.877
2,4-D	3	**2.864	**1145.410	**0.001	**0.294
Explant* 2,4-D	9	*1.002	*400.618	**0.004	**0.109
2,4-D* BAP	9	^{ns}0.471	^{ns}188.379	**0.003	**0.089
Explant* BAP** 2,4-D*	27	**1.83	**732.129	**0.003	**0.233
Error	192	0.535	214.160	0.000	0.012

**p<0.01, * p<0.05, ns: insignificance

The interaction effect of explant type and different levels of BAP

The investigation of the interaction effect of the explant type and different levels of BAP on callus induction showed that, in leaf explant, these two traits (number of callus and relative frequency) did not have a statistically significant difference with increased concentration in the whole explants, although, callus diameter was decreased. Similarly, in stem explant, callus diameter was reduced by increasing concentration, and a significant difference was observed as well. By increasing concentration, there was no significant difference in callus number and relative frequency in root explant, but the traits being studied were significant in dry weight and callus diameter. In dry weight, there was no significant difference, but the traits were significant in callus diameter. In root explant, the highest number of callus was with 0.25 mg/L BA and BAP was 0.156%. In this explant, the highest relative frequency was with 0.25 mg/L BAP was 72.8%, and in root explant, the lowest relative frequency was with 1 mg/L BAP was observed as 8.750%.

In root explant, the highest dry weight was 0.092 g in the hormonal concentration of 0.25 mg/L, and the lowest one was observed as 0.001 g at 0.25, 1, and 2 mg/L hormonal concentration in leaf explant. The largest callus diameter was 1.585 cm at 0.25 mg/L hormonal concentration in root explant, and the smallest one was 0.056 cm at 1 mg/L hormonal concentration in stem explant. The color was dark brown in the explants of leaf and stem. In root explant, 0.25 and 1 mg/L concentrations were gold and 0.50 and 2 mg/L concentrations were light browns. In hypocotyl explant, callus color was gold at 0.50 mg/L concentration and light green at 0.25 mg/L and dark green in other concentrations. Generally, callus color was light brown at the levels of 0.25, 0.5, and 1 mg/L BAP and dark brown at 2 mg/L of this hormone (Table 2).

Table 2. The Interaction Effect of the Explant Type and Different Levels of BAP on Callus Induction and Some Traits

Explant	2,4-D (mg/L)	Number of Callus	relative frequency of callus	Dry weight of callus (g)	Callus diameter (cm)	Callus color
Leaf	0.25	^{a}0.672	^{a}13.438	^{f}0.001	^{f}0.169	dark brown
	0.50	^{a}1.078	^{a}21.563	^{d}0.044	^{fg}0.161	dark brown
	1.00	^{a}0.609	^{a}12.188	^{f}0.001	^{gh}0.084	dark brown
	2.00	^{a}0.516	^{a}10.313	^{f}0.001	^{h}0.065	dark brown
Stem	0.25	^{a}0.156	^{a}23.125	^{f}0.002	^{f}0.180	dark brown
	0.50	^{a}0.891	^{a}17.813	^{ef}0.004	^{f}0.164	dark brown
	1.00	^{a}0.438	^{a}8.750	^{ef}0.002	^{h}0.056	dark brown
	2.00	^{a}0.500	^{a}10.000	^{f}0.002	^{h}0.080	dark brown
Root	0.25	^{a}3.614	^{a}72.813	^{a}0.092	^{a}1.585	gold
	0.50	^{a}3.438	^{a}68.750	^{b}0.062	^{b}0.967	light brown
	1.00	^{a}3.063	^{a}61.250	^{c}0.053	^{b}0.916	gold
	2.00	^{a}2.484	^{a}49.688	^{bc}0.058	^{d}0.482	light green
Hypocotyl	0.25	^{a}2.656	^{a}53.125	^{ef}0.002	0.362	light green
	0.50	^{a}2.547	^{a}50.938	^{e}0.009	0.709	gold
	1.00	^{a}2.250	^{a}45.000	^{ef}0.005	^{e}0.350	dark green
	2.00	^{a}2.422	^{a}48.438	^{ef}0.003	^{d}0.457	dark green
LSD		0.510	10.210	0.007	0.076	

Means within a column followed by the same letter are not significantly different at p<0.05

The interaction effect of explant type and different levels of 2, 4-D

The investigation of the interaction effect of explant type and different levels of 2, 4-D hormone indicated that the number of callus and its relative frequency was reduced by increasing 2, 4-

D in leaf explant, and the significant difference was observed in callus diameter (Table 3). In stem explant, high 2, 4-D caused a significant difference in callus diameter; however, there was no significant difference in the number of callus, relative frequency, and dry weight. In root explant, high 2, 4-D hormone resulted in a significant difference in all traits. In hypocotyl explant, the significant difference was observed by increasing 2, 4-D in callus diameter, but callus number, the relative frequency, and dry weight did not have a significant difference. The highest number of callus was 3.65% at 0.1 mg/L hormonal concentration in root explant, and the lowest one was observed as 0.48% at 0.4 mg/L in leaf explant. The highest relative frequency was 73.12% at 0.1 mg/L hormonal concentration in root explant, and the lowest one was 9.68% at 0.4 mg/L in leaf explant.

The highest dry weight with 0.3 mg/L hormonal concentration was 0.092 g in root explant, and the lowest one was observed as 0.100 g at 0.2 and 0.3 mg/L in leaf explant. The largest callus diameter with 0.1 mg/L hormonal concentration was 1.093 cm in root explant, and the smallest one was achieved as 0.049 cm with .03 mg/L in leaf explant. The color of callus was dark brown in leaf and stem explants, gold with 0.1 and 0.2 mg/L 2, 4-D in root explant, and light brown at 0.3 and 0.4 mg/L concentrations.

In hypocotyl explant, callus color was light green at 0.1 and 0.2 mg/L concentrations, and the 0.3 and 0.4 mg/L were dark green. In general, all callus color was light brown in the whole levels of 2, 4-D.

Table 3. The Interaction Effect of the Explant Type and Different Levels of 2, 4-D on Callus Induction and Some Traits

Explant	2,4-D (mg/L)	Number of Callus	relative frequency of callus	Dry weight of callus (g)	Callus diameter (cm)	Callus color
Leaf	0.100	^{e}1.203	^{e}24.063	^{e}0.002	^{gh}0.134	dark brown
	0.200	^{fg}0.656	^{fg}13.125	^{e}0.001	^{gh}0.162	dark brown
	0.300	^{fg}0.531	^{fg}10.625	^{e}0.001	^{i}0.059	dark brown
	0.400	^{g}0.484	^{g}9.688	^{d}0.043	^{gh}0.134	dark brown
Stem	0.100	^{ef}1.000	^{ef}20.000	^{e}0.001	^{hi}0.089	dark brown
	0.200	^{fg}0.594	^{fg}11.875	^{e}0.003	^{g}0.169	dark brown
	0.300	^{efg}0.766	^{efg}15.313	^{e}0.003	^{ghi}0.119	dark brown
	0.400	^{fg}0.625	^{fg}12.500	^{e}0.002	^{ghi}0.104	dark brown
Root	0.100	^{d}3.656	^{a}73.125	^{b}0.067	^{a}1.093	gold
	0.200	^{ab}3.344	^{ab}66.875	^{c}0.058	^{ab}1.084	gold
	0.300	^{bc}3.016	^{bc}60.313	^{a}0.092	^{b}1.008	light brown
	0.400	^{cd}2.609	^{cd}52.188	^{d}0.047	^{c}0.766	light brown
Hypocotyl	0.100	^{d}2.328	^{d}46.563	^{e}0.004	^{d}0.575	light green
	0.200	^{d}2.469	^{d}49.375	^{e}0.006	^{e}0.485	light green
	0.300	^{cd}2.672	^{cd}53.438	^{e}0.004	^{de}0.502	dark green
	0.400	^{d}2.406	^{d}48.125	^{e}0.006	^{f}0.317	dark green
LSD		0.510	10.210	0.007	0.076	

Means within a column followed by the same letter are not significantly different at p<0.05

The interaction effect of different levels of BAP and 2, 4-D

The investigation of the interaction effect of BAP and 2, 4-D on callus induction demonstrated that high hormonal concentration of BAP and 2, 4-D did not lead to significantly different in the number of callus, and relative frequency. However, there was a significant difference in dry weight and callus diameter (Table 4).

Table 4. The Interaction Effect of Different Levels of BAP and 2, 4-D on Callus Induction and Some Traits

BAP (mg/L)	2,4-D (mg/L)	Number of Callus	relative frequency of callus	Dry weight of callus (g)	Callus diameter (cm)	Callus color
0.25	0.100	^{a}2.125	^{a}42.500	^{fg}0.012	^{be}0.551	light brown
	0.200	^{a}2.188	^{a}43.750	^{de}0.020	^{ab}0.615	light brown
	0.300	^{a}2.047	^{a}40.938	^{b}0.036	^{b}0.562	light brown
	0.400	^{a}1.766	^{a}35.313	^{c}0.029	^{b}0.567	light brown
0.50	0.100	^{a}2.547	^{a}50.938	^{bc}0.031	^{a}0.651	light brown
	0.200	^{a}1.891	^{a}37.813	^{efg}0.013	^{cd}0.475	light brown
	0.300	^{a}1.875	^{a}37.500	^{ef}0.018	^{bc}0.550	light brown
	0.400	^{a}1.641	^{a}32.813	^{a}0.056	^{gh}0.326	dark brown
1.00	0.100	^{a}1.565	^{a}33.125	^{fgh}0.011	^{def}0.408	light brown
	0.200	^{a}1.563	^{a}31.250	^{cd}0.026	^{de}0.430	gold
	0.300	^{a}1.703	^{a}34.063	^{ef}0.018	^{fgh}0.335	light brown
	0.400	^{a}1.438	^{a}28.750	^{h}0.005	^{ij}0.232	light brown
2.00	0.100	^{a}1.859	^{a}37.188	^{de}0.019	^{hi}0.280	light brown
	0.200	^{a}1.422	^{a}28.438	^{gh}0.008	^{efg}0.379	light brown
	0.300	^{a}1.359	^{a}27.188	^{c}0.028	^{ij}0.231	light brown
	0.400	^{a}1.281	^{a}25.625	^{gh}0.007	^{j}0.195	light brown
LSD		0.510	10.210	0.007	0.076	

Means within a column followed by the same letter are not significantly different at p<0.05

The process of callus induction in the explants

The calluses color of the explants was dark brown (Figure 1). Statistically, the highest number of callus was observed on average in root explant (3.5 calluses in Petri), and then it was occurred in hypocotyl (2.469 calluses in Petri) and stem and leaf (0/74 callus in Petri), respectively. Moreover, the number of callus was not significantly different in stem and leaf explants (Figure 1).

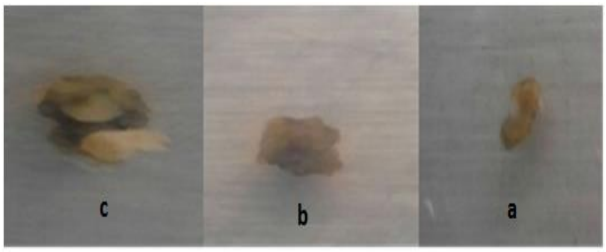

Figure 1. The Process of Callus Induction in Leaf Explant of *Hypericum Perforatum L*,

a. The Callus of Leaf Explant after 7 Days, b. Leaf Callus after 14 days, c. Leaf Callus at the end of 20 Days

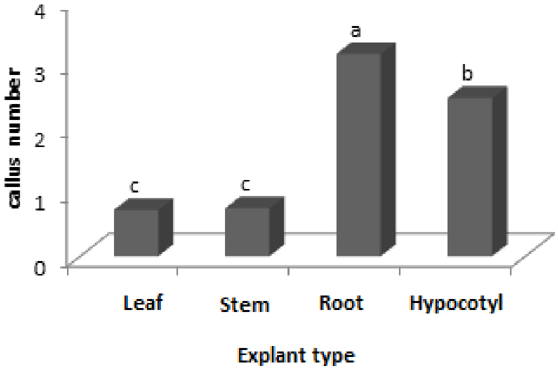

Figure 2. The Effect of Explant on Callus Number of *Hypericum Perforatum L*
Means within a Column Followed by the Same Letter Are Not Significantly Different at $p<0.05$

Calluses color of stem explant was dark brown, which different hormonal concentrations are involved in this process (figure 3). Statistically, root explant with 63% had the highest relative frequency of callus, and it was 49% and 14.9% in hypocotyl and the explants of stem and leaf, respectively. Moreover, there was no significant difference between leaf and stem explants for these traits (Figure 4).

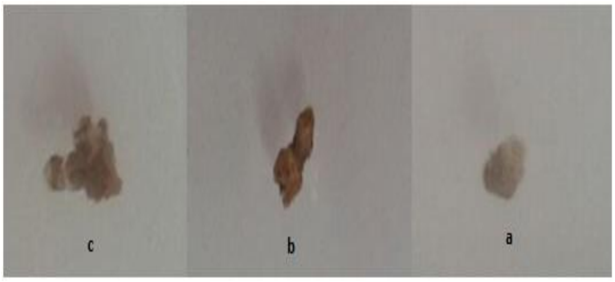

Figure 3. The Process of Callus Induction in Stem Explant of *Hypericum Perforatum L*,
a. The Callus of Stem Explant after 7 Days, b. Stem Callus after 14 days, c. Stem Callus at the end of 20 Days

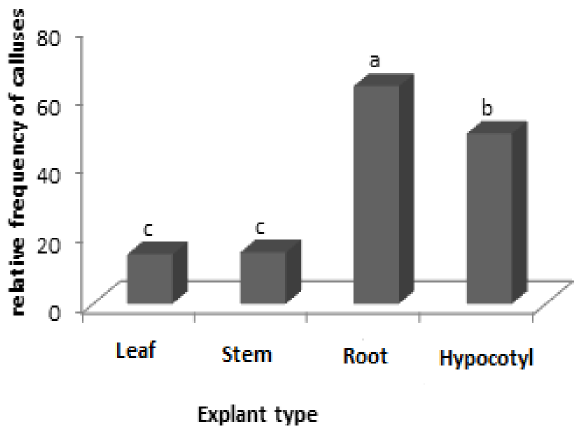

Figure 4. The Effect of Explant Type on Relative Frequency of *Hypericum Perforatum L.* Callus

Means within a Column Followed by the Same Letter Are Not Significantly Different at $p<0.05$

Calluses color of root explant was light brown (figure 5). The highest dry weight of callus with 0.06 g belonged to root explant and hypocotyl with a callus weight of 0.005 was the next one, and stem and leaf explants were not significantly different (Figure 6).

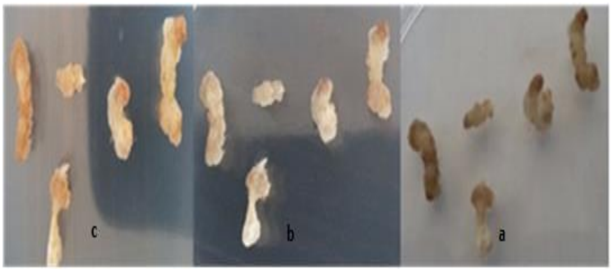

Figure 5. The Process of Callus Induction in Root Explant of *Hypericum Perforatum L*,
a. The Callus of Root Explant after 7 Days, b. Root Callus after 14 days, c. Root Callus in the end of 20 Days

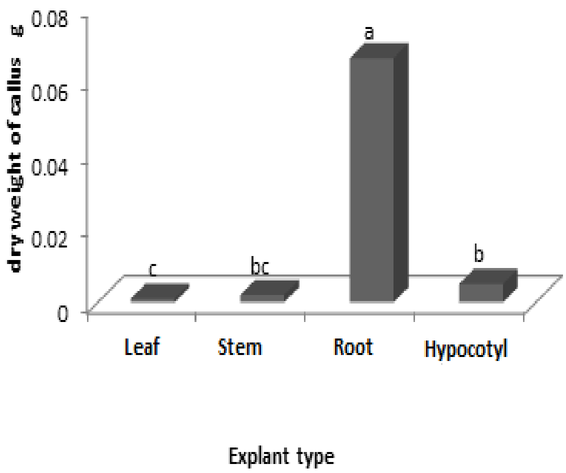

Figure 6. The Effect of Explant Type on Dry Weight of *Hypericum Perforatum L.* Callus
Means within a Column Followed by the Same Letter Are Not Significantly Different at $p<0.05$

Callus color of hypocotyl explant was light green (Figure 7). As it is observed in Figure 8, the largest callus diameter with 0.988 cm was in root explant. With 0.47 cm callus diameter, hypocotyl explant was in the second rank, and it was 0.12 cm in the stem, and leaf explant was not significantly different.

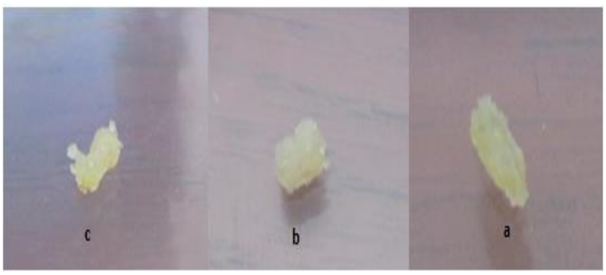

Figure 7. The Process of Callus Induction in Hypocotyl Explant of *Hypericum Perforatum L*,

a. The Callus of Hypocotyl Explant after 7 Days, b. Hypocotyl Callus after 14 days, c. Hypocotyl Callus at the end of 20 Days

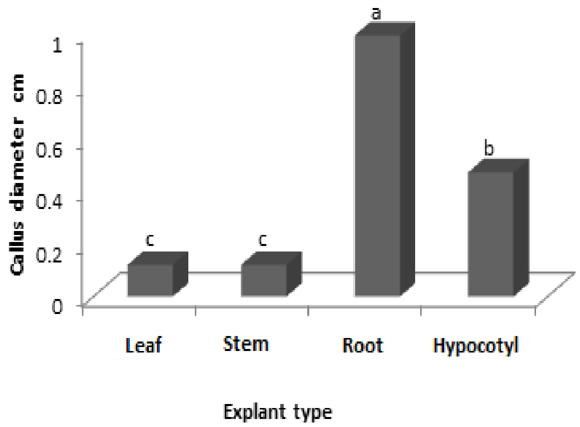

Figure 8. The Effect of Explant Type on Callus diameter of *Hypericum Perforatum L.*
Means within a Column Followed by the Same Letter Are Not Significantly Different at $p<0.05$

Conclusion

Statistically, a high hormonal concentration of BAP caused a decreased number of callus and relative frequency in each of the explants (Table 2), associating with the results of Wójcik and Podstolski (2007). In their studies on callus induction with hormonal treatments of BAP (0.7, 1.7, 2.7) and NAA (0.5, 1, 2.2) carrying out in explants of leaf, stem, and root, a high hormonal concentration of BAP resulted in the low number of callus and relative frequency in diagrams.

According to the statistic, the number of calluses and their relative frequency was decreased by a high hormonal concentration of 2, 4-D in each of the explants, corroborating the ideas of Fabiane and Eliane (2000). They set out a study to examine the callus and regeneration of *Hypericum Perforatum L.* with hormonal treatments of BA (0.1, 1) and 2, 4-D (0.1, 1) mg/L on leaf and root explants in dark condition. The results showed that the relative frequency was decreased by increasing the hormonal concentration from 0.1 to 1 mg/L. According to the results of this research, a high hormonal concentration of 2, 4-D led to decreased callus diameter. This did not support the findings of Fabiane and Eliane (2000). In their results, which was done with 2, 4-D (0.1, 1), callus diameter was not changed by increasing 2, 4-D concentration and it was even increased in some cases. Furthermore, a high concentration of 2, 4-D resulted in an increased dry weight of callus. Some cases were concluded as following after doing statistical analysis, fitting data with each other, developing figures, tables and investigating them:

The results indicated that root with 0.5 mg/L BAP and 0.1 mg/L 2, 4-D was the best explant among leaf, stem, root, and hypocotyl, and the lowest one was in the stem with 0.5 mg/L BAP and 0.2 mg/L 2, 4-D.

It is likely that these results depended on the hormones concentration and their type. It is recommended that these two mentioned cases should be taken into account in the research of tissue culture and genetic. With regard to the fact that each one of the undifferentiated plant cells was able to change to a complete plant, the technique of tissue culture and genetic provided the new insight for biological scientists and researchers. This study was designed to determine the best explant and hormonal levels using different growth concentrations and explant type in order to obtain the highest number of callus to genetic resources of *Hypericum Perforatum L.* The genetic resources of plants are as a valuable asset for human and to meet his/her needs and one of the main factors of development. Therefore, conservation and practical use of them would have a significant position in the sustainable development. Tissue culture instigates with the selection of the desired plant, then the plant tissues are removed from the plant in sterile conditions and the diseased parts of the plant including leaves infected with viral and fungal diseases are removed. This is essential for achieving healthy plants. Numerous parts of the plant including root, stem, leaf, and hypocotyl and other plant tissues can be used for cultivation.

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