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AN INVESTIGATION ON CALLUS FORMATION STAGES IN ST. JOHN'S WORT, *HYPERICUM PERFORATUM L.,* IN CULTURE MEDIUM

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ABSTRACT

St. John's wort is a valuable medicinal plant that has been used for more than 2000 years and is known globally as one of the best medicines in the context of neurological diseases and skin health. As the leading sciences worldwide, plant tissue culture and micropropagation by genetic engineering methods are important indicators of agricultural biotechnology. The fact that undifferentiated plant cells can potentiallyturn into a complete plant can lead to the conclusion that a new gateway has been opened for biologists. As such, it considerably accelerates the time for the implementation of breeding programs in comparison to traditional plant breeding methods and allows inter-genera crosses in plants. In addition, other basic applications of tissue culture by plant microsamples include maintenance of genetic reserves, production of virus-free plants, and paying economic and practical attention to the production of haploid plants. The present research briefly investigates on callus formation stages of St. John's wort in culture medium (MS).

Keywords: culture medium, microsample, callus, St. John's wort (*Hypericum perforatum*)

Introduction

In recent years, St. John's wort has widely been cultivated in Western Europe (Avato and Guglielmi, 2004). This plant, also known with various other names such as Hufarighon and tea grass, is perennial and herbaceous with opposite, oval, slightly elongated leaves (Cui *et al*., 2010). There are two types of dark and bright spots on the leaves, with the latter being scattered all over the leaf surface as the accumulation site for essential oils. The dark spots exist on the margins of the leaves and are the accumulation site of hypericin (the active ingredient of the plant) (Wilhelm *et al*., 2001). There are more than 469 species in the genus *Hypericum* globally, 17 of which have so far been reported from Iran. In many countries, different species of this plant are used as a healing agent and have different medicinal properties. St. John's wort is traditionally and modernly used to treat neurological disorders and depression (Crockett, 2010).

Little progress has been made in the improvement of medicinal plants due to their ancient origin. In recent years, plant raw materials required by the pharmaceutical industry have been harvested from nature, which in addition to the increasing destruction of forests, pastures and green space, has led to the production of heterogeneous raw materials and potential extinction of the species (Pundir and Jain, 2010).

Many wild medicinal and aromatic species are subjected to the risk of extinction and erosion due to over harvesting and destruction of natural habitats. The growing global demand for these species has increased the need for their domestication and cultivation in agricultural systems (Smith *et al*., 2015). Therefore, the cultivation of these plants in agricultural systems seems to be an important strategy in the provision of a growing, wide spread global market for medicinal plants (Uniyal *et al*., 2002). Some of these plants have limited natural habitats and their collection is difficult depending on the environmental and geographical conditions of the plant habitat. Since the use of medicinal plants has a rich and glorious history, this traditional knowledge should be turned into a practical knowledge according to up-to-date standards in order to meet the current needs of the world with scientific language and acceptable to medical and industrial authorities. In this regard, cultivation and domestication of medicinal plants are of particular importance to improve the quantitative and qualitative traits of plant products and produce genotypes with desirable traits (Fotovati and Noorbakhsh, 2009).

Tissue culture has provided a tool for the rapid propagation of a large number of uniform plants while maintaining their genotypes. Concerning medicinal plants, tissue culture and micropropagation methods are used by researchers to obtain a bulk of quasi-original and healthy plants with a high production capacity. Plant tissue culture can be a proper substrate for the conservation of original or endangered species and genotypes in the nature as valuable sources of germplasm (Arikat *et al*., 2004).

Callus culture is generally referred to the formation of undifferentiated cell masses from in vitro tissue culture of a specific species. In addition to plant propagation, plant callus tissue has a variety of other capabilities, including their use in gene transfer to plants or in the preparation of cell suspension cultures to produce secondary metabolites or other useful compounds derived from them (Vasconsuelo and Boland, 2007).

The in-glass culture innovation can be divided into medium- and long-term section. In the medium term, the plant grows in the glass under controlled environmental conditions. In this method, plant growth inside the glass is decelerated by making changes in the environmental conditions or the culture medium ingredients to reduce the number of times required for its propagation. However, the main problem of this method is the need for considerable equipment and facilities to control the environmental conditions required by the plant, which will particularly be problematic with a high volume of stored germplasm through increasing the required costs (for equipment) and manpower (for propagation) (Reinirid *et al*., 1993**)**. The growing use of chemical drugs will cause increasing acute problems such as autoimmune phenomenon due to continuous use and side effects of some drugs. The resistance of pathogens to chemical drugs and their unwanted side effects have led to interests in the use of plant extracts and plants with antimicrobial activity in recent years.

Materials and Methods

Step 1: Immediately after preparation from the Faculty of Agriculture, Ferdowsi University of Mashhad, *H. perforatum* seeds were stored in paper bags in a cold environment.

Step 2: To prepare sterile seedlings, *H. perforatum* seeds were sterilized in 1.5% sodium hypochlorite for 15 min and then washed 3 times with sterilized distilled water in the laboratory of tissue culture and plant physiology of Payame Noor University, Mashhad.

Step 3: To prepare sterile plants for obtaining micro samples, *H. perforatum* seeds were placed in vessels containing sucrose-free MS medium (Murashige and Skoog, 1962).

Step 4: The micro samples used in this experiment included leaves, stems, roots, and hypocotyl. To obtain micro samples, these organs were placed in vessels containing culture medium inside a growth chamber. In this study, hypocotyl microsamples were used after 7 days of seed germination and those of leaf, root, and stem after 45 days of germination.

Step 5: For callus formation, micro samples were cultured in 16 hormonal treatments, namelyat four hormonal levels of benzyl aminopurine (BAP) (0.25, 0.5, 1, and 3 mg/l) and dichlorophenoxy acetic acid (D-2,4) (0.1, 0.2, 0.3, and 0.4mg/l)with sucrose in a factorial experiment based on a completely randomized design with four replications each containing five microsamples (Table 1) and kept in the dark at 25 ± 2°C for 20 days.

Step 6: Then, callus formation stages in leaf, hypocotyl, root, and stem microsamples were evaluated and calculated in a period of 7-20 days.

Table 1 : Different hormonal treatments for callus formation in 16 treatments 2,4-D (0/1, 0/2, 0/3, 0/4) mg/L, BAP(0/25, 0/5, 1, 2) mg/L

Materials required for 50 cc Culture medium for fine sample callus.	**Repeat 4**	**Repeat 4**	**Repeat 4**	**Repeat 4**
	0/25BAP+ 0/1 2,4-D	0/25BAP+0/2 2,4-D	0/25BAP+0/3 2,4-D	0/25BAP+0/4 2,4-D
MS	0/22gr	0/22gr	0/22gr	0/22gr
Sucrose	1.5 gr	1.5 gr	1.5 gr	1.5 gr
2,4D	5ul	10 ul	15 ul	20 ul
BAP	12ul	12 ul	12 ul	12 ul
Ph	5/8	5/8	5/8	5/8
With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc
Agar	0/4 gr	0/4 gr	0/4 gr	0/4 gr
	0/5BAP+ 0/1 2,4-D	0/5BAP+0/2 2,4-D	0/5BAP+0/3 2,4-D	0/5BAP+0/4 2,4-D
MS	0/22gr	0/22gr	0/22gr	0/22gr
Sucrose	1.5 gr	1.5 gr	1.5 gr	1.5 gr
2,4D	5ul	10ul	15 ul	20 ul
BAP	25ul	25 ul	25 ul	25 ul
Ph	5/8	5/8	5/8	5/8
With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc
Agar	0/4 gr	0/4 gr	0/4 gr	0/4 gr
	1BAP+ 0/1 2,4-D	1BAP+0/2 2,4-D	1BAP+0/3 2,4-D	1BAP+0/4 2,4-D
MS	0/22gr	0/22gr	0/22gr	0/22gr
Sucrose	1.5 gr	1.5 gr	1.5 gr	1.5 gr
2,4D	5ul	10 ul	15 ul	20 ul
BAP	50ul	50 ul	50 ul	50 ul
Ph	5/8	5/8	5/8	5/8
With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc
Agar	0/4 gr	0/4 gr	0/4 gr	0/4 gr
	2BAP+ 0/1 2,4-D	2BAP+0/2 2,4-D	2BAP+0/3 2,4-D	2BAP+0/4 2,4-D
MS	0/22gr	0/22gr	0/22gr	0/22gr
Sucrose	1.5 gr	1.5 gr	1.5 gr	1.5 gr
2,4D	5ul	10 ul	15 ul	20 ul
BAP	100ul	100ul	100 ul	100 ul
Ph	5/8	5/8	5/8	5/8
With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc	With a volume of 50 cc
Agar	0/4 gr	0/4 gr	0/4 gr	0/4 gr

Results and Discussion

By the use of Minitab 17.3 software, the interaction of microsample type and different levels of BAP and 2,4-D hormones on callus formation indicated that 0.1mg/l2,4-D + 0.5mg/l BAP and 0.5mg/lBAP + 0.2mg/l2,4-D were the best hormonal treatments in leaves and stems, respectively. The best hormonal treatment in hypocotyl was0.5mg/lBAP + 0.2mg/l 2,4-D. In the root microsample with the best yield among other microsamples, the best hormonal treatment was 0.5mg/lBAP + 0.12 mg/l 2,4-D (Table 2). Results of the best concentrations of hormones for callus formation help us to faster and better achieve our goal by the use of tissue culture technique.

Table 2 : Interaction of microsample type and different levels of BAP and 2,4-D hormones on callus formation and some callus traits

Callus color	Callus diameter (cm)	Dry weight of callus (g)	relative frequency of callus	Number of Callus	(mg/L) 2,4-D	(mg/L)BAP	Explant
dark brown	0/105	0/001	18/750	0/938	0/100	0/25	Leaf
dark brown	0/175	0/001	15/000	0/750	0/200		
dark brown	0/044	0/001	5/000	0/250	0/300		
dark brown	0/351	0/001	15/000	0/750	0/400		
dark brown	0/168	0/003	42/500	2/125	0/100	0/50	
light brown	0/385	0/003	30/000	1/500	0/200		
dark brown	0/009	0/000	2/500	0/125	0/300		
dark brown	0/081	0/169	11/250	0/563	0/400		
dark brown	0/169	0/001	15/000	0/750	0/100	1/00	
dark brown	0/048	0/000	3/750	0/188	0/200		
dark brown	0/037	0/001	20/000	1/000	0/300		
dark brown	0/084	0/001	10/000	0/500	0/400		
dark brown	0/095	0/001	20/000	1/000	0/100	2/00	
dark brown	0/040	0/001	3/750	0/188	0/200		
dark brown	0/108	0/001	15/000	0/750	0/300		
dark brown	0/019	0/000	2/500	0/125	0/400		
dark brown	0/167	0/001	43/750	2/188	0/100	0/25	Stem
dark brown	0/141	0/002	10/000	0/500	0/200		
light brown	0/280	0/004	23/750	1/188	0/300		
dark brown	0/130	0/001	15/000	0/750	0/400		
light brown	0/121	0/003	23/750	1/188	0/100	0/50	
light brown	0/361	0/004	25/000	1/250	0/200		
dark brown	0/014	0/004	2/500	0/125	0/300		
dark brown	0/163	0/004	20/000	1/000	0/400		
dark brown	0/023	0/001	2/500	0/125	0/100	1/00	
dark brown	0/044	0/002	2/500	0/125	0/200		
dark brown	0/073	0/005	20/000	1/000	0/300		
dark brown	0/083	0/002	10/000	0/500	0/400		
dark brown	0/045	0/002	10/000	0/500	0/100	2/00	
dark brown	0/128	0/003	10/000	0/500	0/200		
dark brown	0/107	0/001	15/000	0/750	0/300		
dark brown	0/041	0/000	5/000	0/250	0/400		
Gold	1/461	0/045	65/00	3/250	0/100	0/25	Root
Gold	1/745	0/075	90/000	4/500	0/200		
Gold	1/482	0/137	80/000	4/000	0/300		
Gold	1/651	0/111	56/250	2/813	0/400		
Gold	1/616	0/112	100/000	5/000	0/100	0/50	
light brown	0/669	0/044	52/500	2/625	0/200		
light brown	0/969	0/059	65/000	3/250	0/300		
dark brown	0/613	0/035	57/000	2/875	0/400		
Gold	1/041	0/042	65/000	3/250	0/100	1/00	
Gold	1/244	0/090	75/000	3/750	0/200		
light brown	1/051	0/065	60/000	3/000	0/300		
light brown	0/328	0/015	45/000	2/250	0/400		
dark brown	0/252	0/070	62/500	3/125	0/100	2/00	

Gold	0/677	0/025	50/000	2/500	0/200		
light brown	0/530	0/109	36/250	1/813	0/300		
dark brown	0/470	0/027	50/000	2/500	0/400		
light green	0/473	0/003	42/500	2/125	0/100	0/25	Hypocotyl
light green	0/400	0/003	60/000	3/000	0/200		
light green	0/441	0/001	55/000	2/750	0/300		
dark green	0/135	0/006	55/000	2/750	0/400		
light green	0/699	0/006	37/500	1/875	0/100	0/50	
light brown	0/485	0/003	43/750	2/188	0/200		
light green	1/207	0/010	80/000	4/000	0/300		
light green	0/447	0/018	42/500	2/125	0/400		
dark green	0/400	0/002	50/000	2/500	0/100	1/00	
dark green	0/383	0/013	43/750	2/188	0/200		
dark green	0/180	0/001	36/250	1/813	0/300		
dark green	0/435	0/002	50/000	2/500	0/400		
light green	0/728	0/005	56/250	2/813	0/100	2/00	
dark green	0/671	0/005	50/000	2/500	0/200		
dark green	0/180	0/001	42/500	2/125	0/300		
dark green	0/250	0/001	45/000	2/250	0/400		
	0/153	0/014	20/410	1/020			**LSD**

With the present-time rapid population growth, biotechnology is a science that has a considerable impact on food supply for current and future generations of countries. If our goal is to have valuable, low-cost, and sufficient food security, and we are concerned about the damage to food resources and the coordination of agricultural sectors with the current population growth while most of the agricultural lands and water sources are being used in agriculture, biotechnology will help us in this regard.

In Iran, governmental and academic research institutes, including the University Jihad, are active to increase the level of this knowledge, and acceptable activities have so far been carried out in this field. A clear example is the attention of FAO to the Agricultural Biotechnology Research Institute.

In medicinal plants, paying attention to genetic resources that can be present in hormonal and callus contents play an important role in modern agriculture, particularly in the field of biotechnology, and their protection and practical use will have be of special importance in sustainable development. As one of these facilities, the use of tissue culture method and regeneration of medicinal plants in glass culture medium has been able to propagate extensively important and cost-effective medicinal plants, with completely high propagation rates in comparison to traditional and old indicators.

Medicinal flowers and plants have a special value and importance from the public views, and their production requires costs and the consumption of such inputs as water and fertilizer. Thus, if the present agriculture is updated using novel methods such as tissue culture, it will reduce the costs of water consumption and other inputs, A fundamental step in the direction of Production is high quality and fast.

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