

Study Regarding the in Vitro Regeneration and Use Perspectives in Some *Valeriana officinalis* L. Genotypes

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Abstract

The purpose of the researchers was to evaluate the regeneration possibilities of the species *Valeriana officinalis* L. from the point of view of “in vitro” cultivation of the species, as well as the influence of genotypes and of the culture environment on the “in vitro” reaction of the species. The biological material used in the process of research was made up of cultivated and spontaneous forms of *Valeriana officinalis* L. present in the collection of the University of Agricultural Science and Veterinary Medicine in Cluj-Napoca, Romania. The studied genotypes were: *Valeriana officinalis*, *Volgensis*, *Sambucifolia*, *Collina*, *Rossica*, *Magurele 100*, *Moscova 835* with different geographical provenience. For the “in vitro” culture of *Valeriana officinalis* L. explants stemming from 2-4 week-old plants were used. The explants were formed of fragments of stem measuring 0.2-0.5 cm and leaf fragments 0.4-0.6 cm in length and 0.3-0.5 cm wide. The results obtained were different, according to the hormone balance: callus or direct risogenesis from the inoculated explants. Also we tested the regenerative possibilities from cell suspension from callus and roots with future possibilities for volatile oils extraction from in vitro suspension. The culture environment used was Murashige-Skoog, 1962, with different hormonal variations as follows: V₀ – Mt. Murashige-Skoog culture media; V₁ – 10 mg/L *naftalen acetic acid* (NAA) and 0.1 mg/L benzyl amino purine (BAP); V₂ – 1 mg/L NAA and 1mg/L BAP; V₃ – 0.1 mg/L NAA and 1mg/L BAP.

Keywords: *Valerian*, “in vitro”, hormones, auxines, cytokinines

1. Introduction

The employment of plants in the prevention and treatment of diseases represents the oldest form of known medicine. The experts in the field state that the natural balance of the active elements contained in plants is far less damaging and even more efficient than the synthesis-based medications with a single active ingredient. Nowadays, around the world, about half of the pharmaceutical products are based upon medicinal plants, or have in their composition active ingredients extracted from plants (such as alkaloids, glycosides, essential oils, etc.). (Ciulei et al., 1993; Goina et al., 1967).

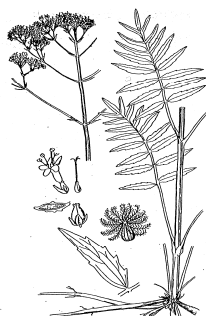


Figure 1. *Valeriana officinalis* L.

One of the plants which ensure the raw material for getting a wide range of drugs is the species *Valeriana*

officinalis L. (Figure1). (www.ro.wikipedia.org/wiki/Valerian%C4%83, <http://www.eukarya.ro/taxon/10210>).

The therapeutic properties of valerian are known since ancient times. Nowadays, the employment of valerian as a sedative and antispasmodic is extending even more. The combination of active elements in the raw material (root stock and roots) of valerian (volatile oils, iridoids, acids, alkaloids, etc) exerts a sedative action on the nervous system and the heart.

The valerian is appreciated as being one of the most important sedatives for the nervous system. It is present in the composition of a large number of extremely important drugs, essential for human health, covering a large spectrum of medical conditions: psychic disorders, heart diseases, conditions of the central nervous system, digestive and muscular diseases, etc. We can also mention other fields where valerian is used: in the food industry (giving flavour to alcoholic and soft drinks, in the preparation of certain meat products, etc.), in the perfume and cosmetic industry, as a decorative plant (some species have red, violet or white flowers), etc. (<http://www.eplante.ro/plante-a-z/odolean-Valeriana-officinalis.html>, http://www.herbmed.org/#param.wapp?sw_page=viewHerb%3FherbID%3D133).

The culture of different explants which contain differentiated cells on aseptic environments rich in auxines leads to cellular mass forming, having an uniform histologic structure. This cellular mass is called callus and it can be cultivated and multiplied through fragmentation and green environmental transfer, maintaining in this way the vitality of the cells and their division capacity. If it's not cultivated on time, the callus grows old and changes it's colour pursuant to metabolite accumulation. According to Nabors, 1983 the callus can be a recuperative type when it leads to complete plant formation or a non-recuperative type when it grows abundantly and it regenerates either roots, either buds, but never complete plants.

The non-recuperative callus has a loose consistency, is brittle, the cells being easily disperseable. Being cultivated on an environment with an over the unit cytokinine/auxine rate, the callus can produce shoots through indirect organogenesis. However we notice that in high concentrations all the auxines can produce callus induction, but in some cases we need less cytokinine for the callus to start forming. This happens because some auxines, when in dense quantities, they can stop the physiologic and morphologic proceses that take place in the plant's cells.

Specific differences exist in the in vitro response of genetically diverse genotypes. Distinctions can be made among cultivars for their callus growth response, ability to initiate shoots and roots, and regeneration potential. Varieties and breeding lines have been identified for their ability to yield numerous regenerated plantlets.

The identification of specific genotypes that are capable of rapid callus production and high rates of plantlet regeneration is an important step toward the application of tissue culture techniques to agriculture (Goldstein & Kronstad, 1985).

2. Materials and Methods

The biological material used in the process of research was made up of cultivated and spontaneous forms of *Valeriana officinalis* L. present in the collection of the University of Agricultural Science and Veterinary Medicine in Cluj-Napoca, Romania.

The studied genotypes were: *Valeriana officinalis*, *Volgensis*, *Sambucifolia*, *Collina*, *Rossica*, *Magurele* 100, *Moscova* 835.

The results obtained were different, according to the hormone balance: callus or direct risogenesis from the inoculated explants.

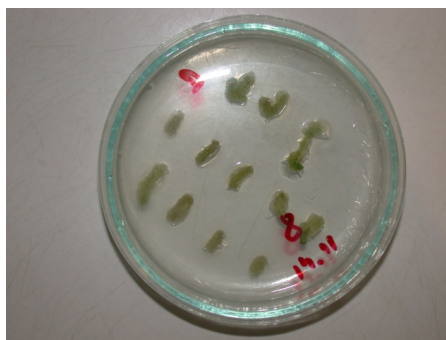


Figure 2. Explants inoculation



Figure 3. "In vitro" reaction initiation

Table 1. MS culture media composition (Murashige & Skoog, 1962)

Constituents			
Macroelements (mg/L)		Microelements (mg/L)	
KNO ₃	1900	CuSO ₄ x 5 H ₂ O	0.025
NH ₄ NO ₃	1650	CoCl ₂ x 6 H ₂ O	0.025
CaCl ₂ x 2 H ₂ O	440	FeSO ₄ x 7 H ₂ O	27.8
MgSO ₄ x 7 H ₂ O	370	Na ₂ EDTA	37.3
KH ₂ PO ₄	170	Vitamins (mg/L)	
ZnSO ₄ x 7 H ₂ O	8.6	myo-Inositol	100
MnSO ₄ x 4 H ₂ O	22.3	Thiamina HCl	0.1
H ₃ BO ₃	6.2	Piridoxina HCl	0.5
KI	0.83	Acid nicotinic	0.5
Na ₂ MoO ₄ x 2 H ₂ O	0.25	Glicina	2
		Saccharose	30 000
		Agar	6700
	pH	5.4 – 5.6	

The culture environment used was Murashige-Skoog, 1962, (Table 1), with different hormonal variations as follows: V₀ – Mt. Murashige-Skoog environment; V₁ – 10 mg/L ANA and 0.1 mg/L BA; V₂ – 1 mg/L ANA and 1mg/L BA; V₃ – 0.1 mg/L ANA and 1mg/L BA. The steps of “*in vitro*” cultivation: A. The sterilization of the vegetal material was performed through the immersion for 10 minutes in a solution of sodium hypochlorite (10 %) followed by three-minute washings in sterile distilled water; B. The inoculation of the plants (Figure 2) was done immediately after they have been measured with sterile instruments; C. The incubation of the explants (Figure 3) was done in a grow room under controlled conditions of temperature (25°C by day and 22°C by night) and illumination (photoperiod of 16 hours of light and 8 hours of darkness). During the first days after inoculation a selection was made, removing the infested containers.

In general, the possibility of applying such methods depends on the species, the donor material, age of the explants or the environment’s formula (Lühns & Lörz, 1987; Nabors et al., 1983; Popelka & Altpeter, 2001; Maës et al., 1996; Dahleen, 1999), (Lühns & Lörz, 1987; Barro et al., 1999, Thomas & Scott, 1985; Maës et al., 1996, as cited in Gana, 2010).

3. Results

Four genotypes of *Valeriana officinalis* L. have been used: *Volgensis* x *Magurele* 100, *Sambucifolia*, *V. officinalis* - *Volgensis*. Also, we have used two types of explants: explants prevailed from the stem and explants prevailed from the leaf blades (about 0.5 cm in length). Inside each variant a spontaneous variant of *V. officinalis* was chosen for comparison with the other genotypes.

Table 2. Variance analysis for V₀ hormonal variant at 14 days from inoculation in 4 valeriana studied genotypes using leaf explants

The variation source	SP	GL	S ²	F	F crit
Genotypes	47.51	3	15.83	0.78	4.75
Repetitions	32.08	2	16.04		
Error	120.42	6	20.07		
Sum	200.02	11			

Applying the 'ANOVA' test (Ciulca, 2006) for the hormonal variation V_0 there are no significant variations to be noted in the results, ($F=0.78 \leq F_{crit}=4.75$) between the valerian genotypes experimented on in order to determine the reaction to the "in vitro" culture (Table 2). This fact can be due to the culture environment used, one without growth hormones.

Table 3. The significance of differences between different genotypes regarding the reaction at "in vitro" culture on V_1 hormonal variant on 14 days from inoculation for leaf explants

Genotype	Mean (%)		Mean difference	Significance
<i>Volgensis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	88.20	77.59	10.61	***
<i>Volgensis</i> - <i>Sambucifolia</i>	88.20	78.51	9.69	**
<i>Volgensis</i> - <i>V.officinalis</i>	88.20	79.86	8.34	**
<i>V.officinalis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	79.86	77.59	2.27	-
<i>V.officinalis</i> - <i>Sambucifolia</i>	79.86	78.51	1.35	-
<i>Sambucifolia</i> - <i>Volgensis</i> X <i>Magurele</i> 100	78.51	77.59	0.92	-

DL 5% DL 1% DL 0.1 %
4 5.56 9.71

From the comparison of the genotypes from the point of view of their capacity to respond to the conditions of "in vitro" conditions of the explants prevailed from leaves, 14 days after the inoculation on the hormonal variant V_1 we have observed the fact that all genotypes react positively to the modern possibilities available for cultivations under controlled conditions. Yet, we can remark the *Volgensis* genotype with an over 88% response percentage. The genotype *V. officinalis* used for comparison registered a response rate of 79.86% (Table 3).

Table 4. The significance of differences between different genotypes regarding the reaction at "in vitro" culture on V_2 hormonal variant on 14 days from inoculation for leaf explants

Genotype	Mean (%)		Mean difference	Significance
<i>Volgensis</i> X <i>Magurele</i> 100 - <i>Volgensis</i>	100	87.36	12.64	***
<i>Sambucifolia</i> - <i>Volgensis</i>	100	87.36	12.64	***
<i>V. officinalis</i> - <i>Volgensis</i>	100	87.36	12.64	***

DL 5% DL 1% DL 0.1 %
1.6 2.41 3.87

After the comparative analysis of the four genotypes of valerian under the aspect of the response of the leaf explants after 14 days after inoculation on the hormonal variant V_2 it was noted that valerian is a species which can be cultivated using the modern possibilities made available by the cell and tissue cultures. The genotype *Volgensis* X *Magurele* 100 was outstanding with a maximum response percentage out of the total number of inoculated explants (Table 4).

Table 5. The significance of differences between different genotypes regarding the reaction at “in vitro” culture on V₃ hormonal variant on 14 days from inoculation for leaf explants

Genotype	Mean (%)		Mean difference	Significance
<i>Volgensis X Magurele 100 - Volgensis</i>	100	14.60	85.39	***
<i>Volgensis X Magurele 100 - Sambucifolia</i>	100	85.92	85.39	***
<i>Volgensis X Magurele 100 - V. officinalis</i>	100	100	0	-
<i>V. officinalis - Sambucifolia</i>	100	14.60	85.39	***
<i>V. officinalis - Volgensis</i>	100	85.92	14.07	-
<i>Sambucifolia - Volgensis</i>	85.92	14.60	71.32	***

DL 5% DL 1% DL 0.1 %
 14.67 22.22 35.7

From the comparison of the genotypes from the point of view of the “in vitro” culture conditions of the explants prevailed from leaves 14 days after the inoculation on the hormonal variant V₃ (which implies a hormonal infusion of auxines and cytokines) we observed that each genotype has a positive reaction to the modern possibilities of cultivation under controlled conditions. Yet, the genotypes *Volgensis X Magurele 100* and *V. officinalis* (the genotype for comparison) have given a 100% response, while the *Volgensis* genotype has had the lowest response rate of 14.6 % (Table 5).

Table 6. Variance analysis for V₀ hormonal variant at 14 days from inoculation in 4 valeriana studied genotypes using stem explants

The variation source	SP	GL	S ²	F	F crit
Genotypes	480.72	3	160.24	3.37	4.75
Repetitions	120.35	2	60.17		
Error	284.49	6	47.41		
Sum	885.58	11			

After applying the variance analysis test for the “in vitro” culture conditions we have observed the fact that there are no significant differences between the valerian genotypes used, taking into account the response of the stem explants cultivated on the V₀ hormonal variant (MS environment without a hormonal input). The F test confirms this theory ($F=17.68 \leq F_{critic}=4.75$) (Table 6). We can also observe the fact that exactly like in the case of the leaf explants, the V₀ hormonal variant does not trigger differences between genotypes.

Table 7. The significance of differences between different genotypes regarding the reaction at “in vitro” culture on V₁ hormonal variant on 14 days from inoculation for stem explants

Genotype	Mean (%)		Mean difference	Significance
<i>Sambucifolia</i> - <i>Volgensis</i>	79.12	55.80	23.31	***
<i>Sambucifolia</i> - <i>V. officinalis</i>	79.12	66.38	12.73	**
<i>Sambucifolia</i> - <i>Volgensis</i> X <i>Magurele</i> 100	79.12	69.79	9.32	*
<i>Volgensis</i> X <i>Magurele</i> 100 - <i>Volgensis</i>	69.79	55.80	13.99	**
<i>Volgensis</i> X <i>Magurele</i> 100 - <i>V. officinalis</i>	69.79	66.38	3.40	-
<i>V. officinalis</i> - <i>Volgensis</i>	66.38	55.80	10.58	-

DL 5% DL 1% DL 0.1 %

6.39 9.64 15.49

After the comparative analysis of the four genotypes of valerian from the point of view of the explants prevailed from the stem, 14 days after the inoculation on the hormonal variant V_1 it was ascertained that valerian is a species which can be cultivated by using the modern possibilities made available by the cell and tissue cultures. The genotype *Sambucifolia* was outstanding with a response rate of 79.12% out of the total number of inoculated explants (Table 7). Yet, the response percentage is slightly lower than the one obtained from the leaf explants.

Table 8. The significance of differences between different genotypes regarding the reaction at “in vitro” culture on V_2 hormonal variant on 14 days from inoculation for stem explants

Genotype	Mean (%)	Mean difference	Significance
<i>V. officinalis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	100 36.92	63.07	***
<i>V. officinalis</i> - <i>Volgensis</i>	100 51.85	48.14	***
<i>V. officinalis</i> - <i>Sambucifolia</i>	100 100	0.00	-
<i>Sambucifolia</i> - <i>Volgensis</i> X <i>Magurele</i> 100	100 36.92	63.07	***
<i>Sambucifolia</i> - <i>Volgensis</i>	100 51.85	48.14	***
<i>Volgensis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	51.85 36.92	14.92	***

DL 5% DL 1% DL 0.1 %
5.74 8.68 13.94

From the comparison of the genotypes from the point of view of their capacity to respond to the “in vitro” culture conditions of the explants prevailed from the stem, 14 days after the inoculation on the hormonal variant V_2 (which implies a hormonal infusion of auxines and cytokines) we observed that each genotype has a positive reaction to the modern possibilities of cultivation under controlled conditions. Yet, the genotypes *Volgensis* X *Magurele* 100 and *V. officinalis* (the genotype for comparison), *Sambucifolia* with a 100% response stand out, while the *Magurele* 100 genotype recorded the lowest response rate, 36.92% (Table 8).

Table 9. The significance of differences between different genotypes regarding the reaction at “in vitro” culture on V_3 hormonal variant on 14 days from inoculation for stem explants

Genotype	Mean (%)	Mean difference	Significance
<i>Sambucifolia</i> - <i>Volgensis</i> X <i>Magurele</i> 100	100 63.43	36.56	***
<i>Sambucifolia</i> - <i>Volgensis</i>	100 77.46	22.54	**
<i>Sambucifolia</i> - <i>V. officinalis</i>	100 84.72	15.27	***
<i>V. officinalis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	84.72 63.43	21.28	***
<i>V. officinalis</i> - <i>Volgensis</i>	84.72 77.46	7.26	-
<i>Volgensis</i> - <i>Volgensis</i> X <i>Magurele</i> 100	77.46 63.43	14.02	*

DL 5% DL 1% DL 0.1 %
9.42 14.24 22.88

After the comparative analysis of the four genotypes of valerian from the point of view of the explants prevailed from the stem, 14 days after the inoculation on the hormonal variant V_3 it was ascertained that valerian is a species which can be cultivated by using the modern possibilities made available by the cell and tissue cultures. The genotype *Sambucifolia* was outstanding with a response rate of 100% out of the total number of inoculated explants (Table 9). Yet, the response percentage is slightly lower than the one obtained from the leaf explants.

4. Discussion

If we refer to the variation of the results for the V_0 hormonal variant for leaf explants, after 14 days from the inoculation there are no significant differences between genotypes.

Numerous factors are reported to influence the success of in vitro propagation of different medicinal plants and therefore, it is unwise to define any particular reason for the general micro propagation of medicinal plants (Rout et al., 2000).

The effective response of plantlets to 1 mg/L BAP during the micro propagation of other *Valerianaceae* species (Kaur et al., 1999) and other medicinal plants such as *Echinacea purpurea* L. (Koroch et al., 2002) has also been reported. In respect of rooting of shoots, we observed that rooting had the better response on MS medium without any PGRs compared to MS medium supplemented with 2 mg/L IBA after 21 days. It seems that rooting in absence of auxins may be attributed to endogenous auxin hormones in the plant. We also found similar results to other medicinal plants. (Sudharsan & Hussan, 2003; Lu, 2005).

From the comparison of the genotypes regarding the capacity of response to the “in vitro” culture conditions of the leaf explants, 14 days after the inoculation, we noticed that in the V_1 hormonal variant, all genotypes react positively to the modern cultivation possibilities in terms of controlled conditions. However, we can notice the *Volgensis* genotype, having an over 88% response rate.

Rani et al., (2003), reported that the best medium for callus formation from hypocotyl, cotyledon and root explants of *Withania somnifera* L. achieved on MS medium supplemented with 0.2 mg/L Kin and 2 mg/L 2,4-D as 100%. In another study, 2,4-D (0.5 to 2 mg/L) introduced as one of the most important auxins in callus induction of *V. edulis* sp. *Procea* (Castillo et al., 2000) and *Leonurus heterophyllus* (Yang et al., 2008). In the current study, combination of Kin (0.1 mg/L) and 2,4-D (2 mg/L) for callus induction from leaf and root explants was more effective than other concentrations which were in agreement with these reports.

In the V_2 hormonal variant we notice that the valerian is a species that can be cultivated using the modern possibilities offered by the cell and tissue cultures. We can distinguish the *Volgensis X Magurele* 100 genotype with a maximum response rate from the total number of explants that were inoculated after 14 days using leaf explants.

Somaclonal variation in regenerated plant genotypes has been described repeatedly in other plant tissue culture literature (Larkin et al., 1984). From the genotypes comparison regarding the response capacity to the “in vitro” culture conditions of the leaf explants, 14 days after the V_3 hormonal variant inoculation (meaning a hormonal input of auxins and cytokines) we notice the *Volgensis X Magurele* and *V. officinalis* (a comparison genotype), having a 100% response rate, while the *Volgensis* genotype registers the lowest response rate 14.6%.

Abdi and Khosh-Khui, 2007, stated that, the best results regarding shoot regeneration of *V. officinalis* was occurred on medium containing 1 mg/L BAP and also the numbers of shoots induced per explants were higher in the presence of 1 mg/L BAP in leaf explants. They found that among the cytokinins that they tested, BAP gave better result compared to Kin in shoot induction.

As a result of the variance analysis for the reaction to the “in vitro” culture conditions we notice significant differences between the valerian genotypes used as a response to the stem explants, 14 days after the inoculation in the V_0 hormonal variant. After the comparative analysis of the four valerian genotypes regarding the response of the stem explants 14 days after the inoculation in the V_1 hormonal variant, we can observe that the valerian is a species that can be cultivated using modern possibilities offered by cell and tissue culture. We can distinguish the *Sambucifolia* genotype with a 79.12 % response rate.

Also, in other medicinal species (Lu et al., 2008; Peng et al., 2008). Mehrotra et al., (2008), stated that application of auxins such as IAA in low concentrations (0.1 mg/L) to the medium promoted production of hairy roots of *Glycyrrhiza glabra*.

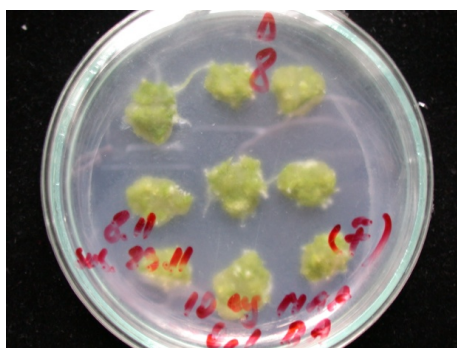


Figure 4. Callus “in vitro” reaction

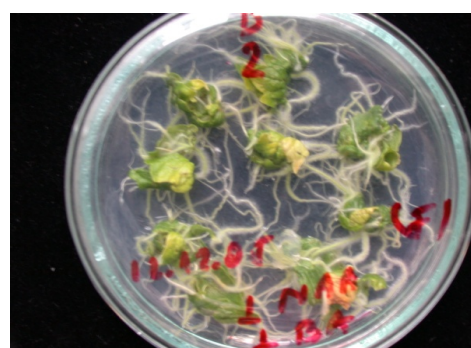


Figure 5. Rooting “in vitro” reaction

After comparing the genotypes as for their response capacity to the “in vitro” culture conditions of the stem explants 14 days after the inoculation in the V₂ hormonal variant (implying a hormonal input of auxines and cytokines) we can notice that all genotypes react positively to the modern cultivation possibilities under controlled conditions. However we can distinguish the *Volgensis X Magurele*, *V. officinalis* (a comparison genotype), *Sambucifolia* having a 100% response rate. After comparing the four valerian genotypes concerning the stem explants 14 days after the inoculation process in the V₃ hormonal variant we notice that the valerian is a species that can be cultivated using modern possibilities offered by the cell and tissue culture. We can distinguish the *Sambucifolia* genotype, having a 100% response rate. According to the hormonal balance scale that has been used we acquired two types of reaction: callus and roots (direct organogenesis). We can exploit this in the future in order to obtain volatile oils through the “in vitro” cultivation techniques, being possible to obtain vegetal biomass under the form of roots (the organs where the volatile oils naturally accumulate to) or using the callus for the induction of suspended cells cultures using bioreactors (Figures 4 and 5).

This makes it interesting to study the possibility of producing secondary metabolites through plant cell and tissue culture (Becker et al., 1977). In the 1990s, increases in yield of valepotriates in transformed roots by *A. rhizogenes* in Valerianaceae were reported frequently (Gränicher et al., 1992 & 1995; Banerjee et al., 1998; Caetano et al., 1999).

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