INDIRECT ORGANOGENESIS OF *SYMPHYTUM OFFICINALE* L.

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Abstract: *Symphytum officinale* L. (Boraginaceae) is a well-known medicinal plant and a source of natural compounds with high antioxidant activity. The initiation of "*in vitro*" cultures of *Symphytum officinale* L aimed not only to assess the dedifferentiation capacity depending on explant origin and growth regulators, but also to develop a multiplication protocol based on indirect regeneration through shoots, followed by roots development induction. The proliferative capacity was tested on leaf and shoots explants, cultivated on Murashige-Skoog basal medium (MS), testing two auxins:: naphtalenacetic acid (NAA) and indolylacetic acid (IAA) and two cytokinines: kinetine (K) and benzylaminopurine (BAP).
The MS medium with 1.0 mg/l IAA and 0,1 mg/l BAP proved to be the best for callus induction from leaf explants. Shoot regeneration was achieved after subculturing the calli on MS medium supplemented with 1 mg/l BAP and 0,1 mg/l IAA. It was found to be the best for multiple shoot regeneration from callus through organogenesis
Multiple shoot proliferation was noticed at 3th subculture in medium and shoot proliferation was decreased with the increased number of subculture. Root system development was achieved on MS medium without growth regulators. Rooted shoots (plantlets) were gradually acclimatized.

INTRODUCTION

Symphytum officinale L. is a perennial plant belonging to the Boraginaceae family. The presence of polyphenols, triterpenoids and tannins in this species represents a promising source of natural compounds with high antioxidant activity (Noorwala et al., 1994).
Many members belonging to the Boraginaceae family, including *S. officinale*, are found to contain the chemical constituent allantoin. Comfrey contains about 0.8% in the root and 0.4% in the leaf .(Winship, 1991).
Like other members the Boraginaceae, the roots of *Symphytum officinale* L contain pyrrolizidine alkaloids, which are hepatotoxic and carcinogenic agents (Winship, 1991). the highest alkaloid levels were found in bulk comfrey roots and leaves (Betz et al. 1994, Frolich et al, 2007, Dreger et al, 2009).
The root extracts of *S. officinale* were tested for their antimitotic and mutagenic activity (Furmanowa et al., 1983, Mei et al, 2005).
"*In vitro*" cultivation of *Symphytum officinale* L was initiated in order to evaluate the cell dedifferentiation and redifferentiation, as an unconvential alternative for plant biomass multiplication, the main source of bioactive compounds with pharmaceutical value (Harris et al, 1989, Huizing et al., 1983, Tacke et al., 1993, Shimon-Kerner et al., 2000).
The objective of the present investigation was to establish in vitro culture and plant regeneration methods from leaf and stem explants of *Symphytum officinale* L.

MATERIAL AND METHODS

The cultures of *Symphytum officinale* L. were based on explants taken from mature individuals, harvested from spontaneous flora.
Foliar and stem explants taken from *Symphytum officinale* L, produced in aseptic conditions were tested " *in vitro*" for proliferative capacity (Haaß et al, 1991).
Callus induction was performed on leaf and shoot explants, cultivated on different variants of MS medium (Table).
The preparation of explants for inoculation was the chemical sterilization, using a solution of 3% Na hypochlorite. Treatment duration was 12 minutes, followed by washing repeatedly with sterile distilled water.
The diversification of MS induction media was based on two types of auxins: naphtalenacetic acid (NAA) and indolylacetic acid (IAA) and two cytokinines: kinetine (K) and benzylaminopurine (BAP).
The biomass accumulation was measured by regular weighting on analytical balance. For callus induction, it were tested some variants of MS medium, using indolylacetic acid (IAA) in combination with benzylaminopurine (BAP).in same concentration (variants 1) or with indolylacetic acid (IAA) in excess (variants 2). The second tested auxin was

naphtalenacetic acid (NAA) used in combination with kinetine (K), in same concentration (variants 4) or in excess (variants 5).
Indirect micropropagation consisted in shoots development from callus cultures., cultivated on different variants of MS medium, supplemented with benzylaminopurine 1 mg/l with 0,1 mg/l indolylacetic acid (IAA) (Table)) or 1 mg/l kinetine with 0,1 mg/l naphtalenacetic acid (NAA) .
All the cultures were maintained at 25^0 C under 12 hr photoperiods.
The cultures growing on various levels of growth regulators were scored for the number of shoots per culture and rooting after every 2 weeks.
The morphogenic response of cultures was followed every 4 weeks by monitoring the fresh weight, number and height of regenerants , as well as root formation.

Table - Variants of MS medium

Variants	Growth regulators			
	BAP	**IAA**	**NAA**	**K**
1	1 mg/l	1 mg/l	-	-
2	0,1 mg/l	1 mg/l	-	-
3	1 mg/l	0,1 mg/l		
4	-	-	1 mg/l	1 mg/l
5	-	-	1 mg/l	0,1 mg/l
6			0,1 mg/l	1 mg/l
7	-	-	-	-

IAA - β indolylacetic acid
NAA - α-naphtalenacetic acid
BAP - benzylaminopurine
K – kinetin

RESULTS AND DISCUSSIONS

The initiation of "*in vitro*" cultures aimed to evaluate the capacity of cell dedifferentiation and redifferentiation at *Symphytum officinale* l., depending on the origin of the explant and hormone stimuli in the culture medium.
The diversification of the composition Murashige – Skoog (Murashige et Skoog, 1962) was based on the use of auxins and cytokinins in various combinations and concentrations.
Among the auxins tested were used: IAA - β-indolylacetic acid and NAA - α-naphthalenlacetic acid and the cytokinins : BAP- benzylaminopurine and K - kinetine.
Testing the proliferative capacity of explants on different types of induction media (Table) aimed in the first instance, to obtain primary callus cultures.
Leaf and stem explants taken from *Symphytum officinale* L, obtained aseptically were tested "*in vitro*" for proliferative capacity.
Callus cultures were periodically transferred every 3 weeks on fresh medium. The next step is to multiply the callus over a period of 6 rounds of subculturing.
The samples were kept at 12 hours light and 12 hours of darkness.
Callus was achieved using surface cultures on agar medium and callus proliferation during several stages of subculturing was assessed by periodic weighting of fresh biomass.
Initiation of "*in vitro*" cultures from *Symphytum officinale* L on the basal MS medium variants (table) generated different reactions, depending on the origin of explant, especially auxins type and hormone balance.
The dedifferentiation has been difficult, in the case of stem explants, while leaf segments were generated primary callus, within 4 weeks after cultures initiation (Photo 1, 2, 3).

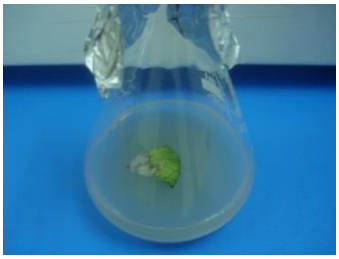

Photo 1- The initiation of callus cultures from foliar explant at *Symphytum officinale* L

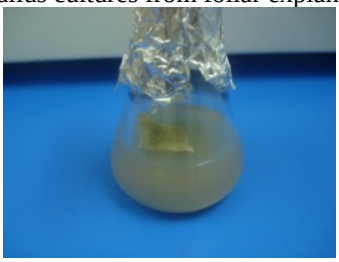

Photo 2- Early stages of cell dedifferentiation at *Symphytum officinale* L.

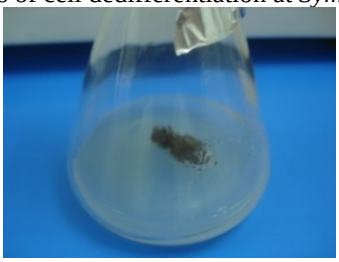

Photo 3- Primary callus culture from foliar explant

Some variation in the frequency of callus formation and in callus growth rate was noted among explants of different origins.
The response of stem explants to IAA or NAA was slower and was initiated only after 6 weeks culture.
Both β-indolylacetic acid (IAA) and α-naphtalencetic acid (NAA), have caused similar effects on leaf explants, completed to the development of primary callus. Excess of auxin in both variants generated callus.
Undifferentiated tissue developed 7-14 days after placement on the medium, at the edges of the cut borders of the foliar explant.
Two weeks later, when calli reached 0,5-1,0 cm diameter, they were cut from the explant and subcultured on medium. Subculturing on medium was done by transferring 6-10 mm diameter pieces of callus onto fresh medium.
Callus of foliar origin was tested for evidence the regenerative capacity.
The growth regulators balance tested in this case is based on the use of cytokinine in higher concentration than auxin. It were tested two cytokinine: BAP – benzylaminopurine and K-kinetine.

The caulogenesis induction was performed on MS medium, supplemented with 1 mg/l BAP – benzylaminopurine in combination with 0,1 mg/l IAA – β-indolylacetic acid (Photo 4, 5, 6).

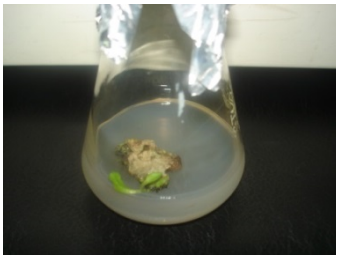

Photo 4 -Early stage of caulogenesis

Photo 5 - First shoot forming on callus culture

Photo 6- Early stages of cell redifferentiation at *Symphytum officinale* L.

Calli with morphogenetic capacity were compact and yellowish to green in colour. Shoot regeneration was always preceded by an early stage of callus growth (Photo 7).

Photo 7- Advanced stages of caulogenesis

Foliar origin calli in the presence of BAP showed the development of multiple shoots, within 4-5 weeks culture. New shoots were continuously formed during the observation period (Photo 8).

Photo 8- Multiple shoot development

Single shoots were excised from 5 week old regenerating cultures and transferred on MS medium for root induction (MS- without growth regulators) (Photo 9).

Photo 9- Regenerated shoot of *Symphytum officinale* L

The time of root initiation and rooting percentage (percentage of shoots forming roots) was determined. A shoot having one or more macroscopically visible roots (0,5 cm long) was considered rooted (Photo 10).

Photo 10- Roots development

Morphogenetic potential expressed in primary callus cultures decreased during the 6 stages of subcultivation. Whole plantlets were transferred to pots containing agricultural substrate and sand (1:1).

CONCLUSIONS

The dedifferentiation has been difficult, in the case of stem explants, while leaf segments were generated primary callus**.**

Both β-indolylacetic acid (IAA) and α-naphtalencetic acid (NAA), have caused similar effects on leaf explants, completed to the development of primary callus.
Foliar origin calli in the presence of BAP showed the development of multiple shoots.
The caulogenesis induction was performed on MS medium, supplemented with 1 mg/l BAP – benzylaminopurine in combination with 0,1 mg/l IAA – β-indolylacetic acid.

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