“IN VITRO” MULTIPLICATION OF *CALENDULA OFFICINALIS* L.

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Abstract:. The aim of the present study was to develop a regeneration procedures for *Calendula officinalis* L., as an alternative for biomass production. *Calendula officinalis* L. (Asteraceae) is an important medicinal plant species with multitherapeutic, cosmetic, values. Meristematic explants taken from seedlings of *Calendula officinalis L.* germinated in aseptic conditions were tested for their regenerative potential. The regeneration of whole plants was obtained in two steps: the shoots were excised and transferred to fresh medium and then rooting of these shoots was achieved on the same medium with 0,02 mg/l benzylaminopurine and 1 mg/l 2,4 dichlorophenoxyacetic acid. The excised shoots were subcultured for roots induction. Regenerated plants were transferred to ex vitro conditions for an acclimatisation period.

INTRODUCTION

Calendula officinalis L. (Asteraceae) is an important medicinal plant, that contains various classes of compounds (volatile oil, carotenoids, flavonoids, terpenoids, coumarins carbohydrates, lipids, aminoacids) with multiple pharmacological activities (Nizynski et al.,2015, Radioza et Iurchak, 2007, Kurkin et Sharova, 2007, Khalid et al.,2012, Ahmad et al.,2012, Herman et al.,2013).
This paper aims to study the behavior 'in vitro' of *Calendula officinalis* L., using an unconventional techniques of clonal micropropagation (Legha et al.,2012).
The morphogenetical potential of meristematic explants from *Calendula officinalis* L. has been set by testing the initiation, multiplication and rooting phases of micropropagation on Murashige –Skoog basal medium with different hormonal balances (Evans, 1990, Edwin et al.,2008).
The multiplication procedure contains the determination of sterilizing agent, the selection of suitable medium for each stage of cultivation.

MATERIAL AND METHODS

The meristematic explants were harvested from Calendula officinalis L. seedlings obtained by germinating the seeds under aseptic conditions. Aseptic preparation of the plant material consisted of chemical treatment with 70% ethanol (one minute) followed by a solution of 10% calcium hypochlorite (for 10 minutes).
The removal of chemical sterilization was carried out by repeated washing of the explant in sterile water.
The reactivity of explants was tested on MS medium Table 1 (Murashige and Skoog, 1962) in different variants, using a cytokinin: benzylaminopurine (1 mg/l, 0,2 mg/l, 0,02 mg/l) and an auxine: 2,4 dichlorophenoxyacetic acid (1mg/l, 0,02 mg/l, 0,05 mg/l).
Each variant differs by the concentration of two types of growth substances and were used not only for caulogenesis, but also for roots development.(Table 2). The samples were maintained in the growth chamber at a constant temperature of 23 ± 1oC and 16 hours photoperiod. The procedure involved shoot tip cultures, followed by rapid shoot multiplication, rooting and finally establishment of plantlets in soil.

Table 1- The composition of Murashige-Skoog medium

	Components	**Concentrations (mg/l)**
Macroelements	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2 \cdot 2H_2O$	440
	$MgSO_4 \cdot 7H_2O$	370
	KH_2PO_4	170

	Components	**Concentrations (mg/l)**
Microelements	H_3BO_3	6,2
	$MnSO_4 \cdot H_2O$	22,3
	$ZnSO_4 \cdot 7H_2O$	8,6
	$Na_2MoO_4 \cdot 2H_2O$	0,25
	$CuSO_4 \cdot 5H_2O$	0,025
	$CaCl_2 \cdot 6H_2O$	0,025
FeEDTA	$FeSO_4 \cdot 7H_2O$	27,8
	Na_2EDTA	37,3
Vitamins	*Acid nicotinic*	0,5
	Piridoxină HCl	0,5
	Tiamină HCl	0,1
	Mezo-inozitol	100
Amino acids	*Glycine*	2
Sucrose		30000
Agar		10

Table 2- Variants of MS- medium

Variants of MS medium	**Growth regulators**	**Concentration mg/l**
I	BAP	1
	2,4 D	1
II	BAP	0,2
	2,4 D	0,05
III	BAP	1
	2,4 D	0,02
IV	BAP	0,02
	2,4 D	1

RESULTS AND DISCUSSIONS

The interest for "in vitro" cultivation of *Calendula officinalis* L. is justified by using an unconventional method to assess the possibilities for direct regeneration (Evans, 1990).

The first stage of the initiation of culture was the chemical sterilization of explants, operation that was completed in a satisfactory 90%, obtaining material under aseptic conditions.

The apical shoot explants, taken from Calendula officinalis L. seedlings were used for direct micropropagation, in two stages: caulogenesis, followed by rhizogenesis. Shoot tips were the source of regeneration in vitro.

Regeneration induction media used for *Calendula officinalis* L. micropropagation were Murashige-Skoog medium (MS), supplemented with two types of growth substances: benzylaminopurine and 2,4 dichlorophenoxyacetic acid (Grzelak et Janiszowska, 2002)

In each variant of MS basal medium it has worked on every 10 samples, corresponding to the four variants. Samples were kept in growth chamber at 23±1o C and a photoperiod of 16 hours.

The research revealed the direct organogenesis, manifested primarily by bud formation, which later gave rise shoots obtained on MS medium (variant II).

The MS medium containing 0,2 mg/l benzylaminopurine and 0,05 mg/l 2,4 dichlorophenoxyacetic acid was optimum for shoot proliferation at.*Calendula officinalis* (Photo 1, 2*)*.

Benzylaminopurine (BAP) in excess stimulated the formation of multiple shoots (Photo 3, 4, 5). The shoots were separated and cultured individually on rooting media.

Roots development was stimulated on variant IV of MS medium , based on a balance with an auxin in excess (1mg/l 2,4 dichlorophenoxyacetic acid). This concentration of auxine has generated the development of the root system at 100% shoots (Photo 6).

Early roots development process was observed after 12 days of placing individual shoot on inductive medium. The plants were regenerated in vitro were transferred into pots with soil mix in order to adapt them to ex vitro life. *Calendula officinalis* L. plants, regenerated 'in vitro' (Photo 7), 8) were acclimatized in ex vitro conditions by transplanting them into pots containing a mixture of peat and perlite (J:1) and maintained in growth chamber at 24 C and 16.

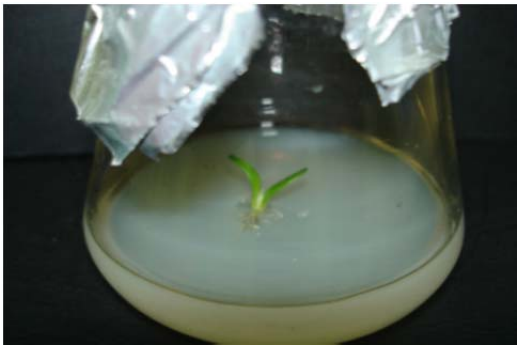

Fig. 1- Initiation of culture at Calendula *officinalis* L.

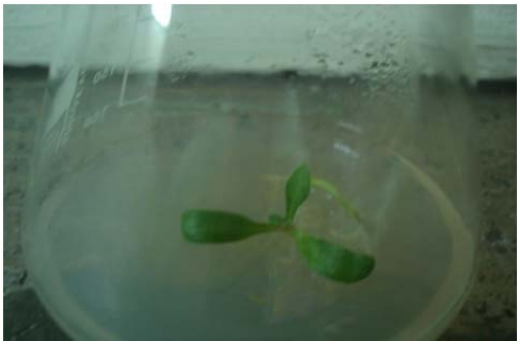

Fig.2- Early stages of caulogenesis

Fig. 3- Multiple shoots development

Fig. 4- Multiple shoots development

Fig. 5- Induction of rhizogenesis

Fig. 6- Roots development

Fig. 7- Acclimatisation of regenerated plants

Fig. 8- Acclimatisation of regenerated plants

CONCLUSIONS

The regenerative potential of meristematic explants from *Calendula officinalis L* was evaluated for the establishment of a clonal propagation protocol.
Plant regeneration has been achieved from meristem multiplication using shoot tips.MS medium has been diversified according to hormonal balance, using benzylaminopurine in combination with 2,4 dichlorophenoxyacetic acid.
The agar solidified MS medium containing 0,2 mg/l benzylaminopurine and 0,05 mg/l 2,4 dichlorophenoxyacetic acid was optimum for shoot proliferation at.*Calendula officinalis L. and* allowed the development of large number of cloned shoots.
Calendula officinalis L. plants, regenerated 'in vitro' were acclimatized in ex vitro conditions.

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