OBSERVATIONS ON THE 'IN VITRO' AND 'EX VITRO' BEHAVIOUR OF *CHRYSANTHEMUM BALSAMITA L.* SPECIES

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Abstract: The paper presents the results of the investigations concerning the "in vitro" culture initiation of *Chrysanthemum balsamita* species, the behaviour of the explants on different nutritive mediums, the obtaining of neoplanlets and their accommodation, the behaviour of regenerants in the field.

AIM OF INVESTIGATIONS

The aim of these investigations was to establish the morphogenetic reaction in "in vitro" cultures of *Chrysanthemum balsamita* (callus cultures, regenaration of neoplantlets, accommodation of regenerants, etc).

INTRODUCTION

Chrysanthemum balsamita L. is a perenial plant belonging to the *Asteraceae* family originating from Southern Asia, brought to Europe since antiquity. In our country it is frequently found in peasants` gardens and in cemeteries, being cultivated as an ornamental and aromatic plant. There are two different morphological and chemical varieties of this species: var. *balsamita* which has ligulated white flowers, 2n=18 chromosomes and in its volatile oil prevails camphor; var. *tanacetoides* that lacks the ligulated white flowers, the number of chromosomes in its somatic cells being 2n=54, in its volatile oil prevails carvone.Using gas-chromatographical studies one rendered evident 80 components in the etheric oil of var. *balsamita* and 103 components in the volatile oil of var. *tanacetoides.* Apart from volatile oil other active principles were identified in flowers and leaves, such as: flavones, phenyl-propanic derivates, carotens, tanins, sescviterpenic lactons (6,7,9). Romanian traditional medicine uses this plant to treat wounds, ulcers in the mouth, tooth aches, lung and liver diseases, to stop bleedings, as a fortifiant for women after birth and for new-born babies etc. Modern medicine enhanced the antibiotic and antipyretic action of volatile oils. Hydro-alcoholic extracts from dry leaves of var. *tanacetoides* are known for their properties in liver protection. Camphor stimulates peripherical blood flow, the simpathetic nervous centres of heart heart, antipruritic and septic effect. Carvon is also known to stimulate CNS, peripherical blood flow and respiratory centres, insecticide effect etc (6,7,9).

Considering the possibilities offered by ″in vitro″ cultures of plants (1,5,8,10), the pharmaceutical importance of this species and the fact that *Chrysanthemum balsamita* breeds vegetatively we considered that it would be convenient to test its behaviour in ″in vitro″ and ″ex vitro″cultures and to take part in elaborating a new multiplication technology through unconventional techniques, as well as isolating some potential somaclonal variations to obtain new genotypes of this species.

MATERIAL AND METHOD

We fulfilled our investigations on the *tanacetoides* variety of the *Chrysanthemum balsamita* species. The source of explants to initiate ″in vitro″cultures were individuals of *C. balsamita* supplied by the Medicinal Plant Laboratory in Braşov, that were cultivated at 'Stejarul' Research Centre in Piatra Neamt in the spring of 1998. The explants used to initiate the 'in vitro' culture were tips of stem shoots (from the floriferous stem) gathered at the end of August 1999. Explants sterilisation was carried out with 0.1% mercury chloride solution (for 12 minutes) or with 5% T-chloramine solution (for 20 to 30 minutes). The explants were then washed twice

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with distilled water and inoculated on Murashige-Skoog (1962) hormone-free medium or that has been supplemented with BAP (0.2-2.0 mg/l), with BAP and IAA (1/1), BAP and NAA (1/1), 2.4-D (2 mg/l). Saccharose (25 g/l) was used as a charbon source for the culture mediums. To solidify mediums we used agar (8.5 g/l). The 'in vitro' cultures were placed in Erlenmayer wide-neck 100 ml phials. Culture incubation was proceeded in a half-climatised room that belongs to 'Stejarul' Research Centre (temperature 23 to 25°C, aproximate light of 2000 lux, continuous lighting).

It has been ascertained that the use of chloramine-T provides a much higher rate of explant survival and the most efficient hormonal formulae to initiate the 'in vitro' culture is to supplement the MS medium with 0.5 to 1.0 mg/l BAP. Good results were also obtained on MS hormone-free medium. The biological material got on the initiation 'in vitro' culture mediums was subsequently used to test the morpho-genetic reaction of various explants on different hormonic formuli (displayed in table 1). The morphogenetic reaction of the tested explants as features of the regenerants' behaviour in field are presented in fig.1.

RESULTS AND DISCUSSIONS

Multi-annual tests for initiating 'in vitro' cultures of *C. balsamita var. tanacetoides* rendered that the most indicated method to sterilize the explants is to treat them with T-chloramine 5% solution for 30 minutes as this method also assures a high percentage of survival for the inoculated biological material. The most favourable medium formulae to resume explants' growth processes used in culture initiation (very young apical and axillary shoots) were MS hormone-free medium or MS medium supplemented with BAP amounts from 0.2 to 1.0 mg/l. On this initiation mediums the inoculated shoot tips produced a compact, small, green callus (resembling a thickened stem) that gave rosette-shaped shoots that had different numbers of leaves within rosettes. The sterile shoots obtained were then used as a source of explants to test their morphogenetic reaction on diverse hormonic formulae of the MS medium, (Table 1).

On MS medium supplemented with BAP, or BAP and IAA, or BAP and zeatine, the rosette-shaped shoots inoculated provided a compact, small size, green callus (as a thickened stem) that produced numerous shoots (from 3 to 50) shaped as multiple rosettes. Callus was better developed on BA medium and the shoots were kind of grown together being more difficult to split than on B medium.

Differences in shoot strength, leaf thickness and colour, leaf shape (some leaves were spear-shaped, others had a wide-tip limb having a small spade aspect) were rendered depending on the hormonal formulae used. During shoots' split inside the generated shrub on the previously mentioned mediums it was ascertained that they synthetize the specific etheric oil, one being able to sense the strong smell of *C. balsamita* oil. The

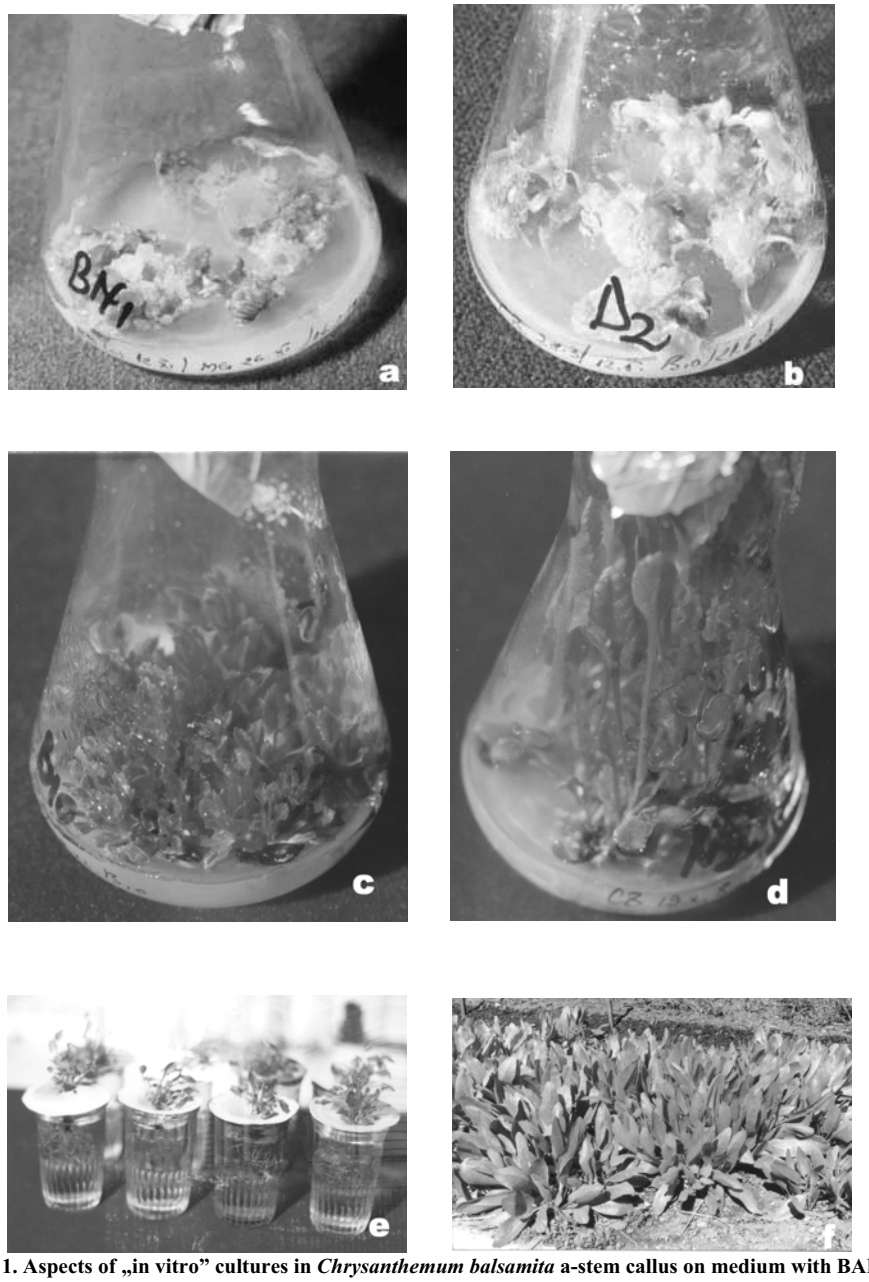

Fig. 1. Aspects of „in vitro" cultures in *Chrysanthemum balsamita* a-stem callus on medium with BAP + NAA; b-stem callus on 2,4-D; c-neoplantlets on BAP; d-neoplantlets on NAA; e-accommodation of regenerants; f-regenerants cultivated in the field

The small rosette-shaped shoots inoculated on A, GA, GN, N and IB hormonic formuli produced neoplantlets, the most vigorous (with numerous and strong roots) being developed on mediums supplemented with 2 mg/l of IAA and NAA. Within a month the shoots reach culture phial height.

There is a high frequency of multiple shooting phenomenon on GA medium absent on A medium and weakly represented on IB medium. If we formed on the hierarchical system the capacity of generating vigorous neoplantlets on the five medium formulae, the succession would be: A>N>IB>GA>GN. On the nutritive medium adding 2 mg/l IAA, the leaves were much wider than on the other hormonal formulae, and on NAA medium with 2 mg/l the roots had the most intense rate of growth, during 6 weeks of subcultivation reaching about 20 cm in length.

On MS hormone-free medium the process of shoot striking roots is less evident than on the above mentioned mediums. Rosette-shaped shoots' inoculation on the medium supplemented with 2 mg/l 2,4-D inhibited shoot growth and at the contact between shoots and the nutritive medium a compact rugged cream-greenish coloured and low proliferation capacity callus appeared. In some cases this callus was friable. Its subcultivation on mediums supplemented with 0.2 to 1.0 mg/l of BAP did not determine cell differentiation and the callus degenerated in time. Its cultivation on the same hormonal formula (D) assurede neither the callus cell proliferation speed nor its outliving, (table 1, figure 1). Little leaves detached from rosettes were also used as explants.

It has been found that on mediums supplemented with 2 mg/l IAA and 1 to 2 mg/l IBA, the leaves grew much in size and produced very strong roots at the sectioned end of the leaf stalk. With the increasing amounts of IBA in the nutritive medium (2 mg/l) on some parts of the petiole a thin layer of white callus formed as well as bunches of fine white roots. On BD medium the leaf lamina grew considerably in thickness, turned to a lighter colour, on some parts of the limb and petiole having a compact, greenish low proliferative callus.

On a medium supplemented with 2 mg/l 2,4-D the leaves generated a friable cream-coloured also low proliferative callus, (table 1). Fragments of roots gathered from the neoplantlets obtained on MS, GA, GN were also used as explants. Their inoculation on medium formulae A, IB and D caused callus generation with a low cell - multiplying rate. If the MS medium was supplemented with 2 mg/l IBA the root callus would be compact, filamentous, brown-greenish. If the medium contained 2 mg/l IAA the callus was friable, cream-greenish and it was produced on the entire root surface.

Supplementing the medium with 2 mg/l 2,4-D the roots gave a friable cream-greenish colour callus on their whole surface. The root callus could not be repeatedly subcultivated and also didn't prove having organogenetic capacity by its transfer on other hormonal formulae.

Repeated shoot cultivation on MS medium supplemented with 1-2 mg/l BAP led to a hyperhydration phenomenon that can be avoided by shoot growth on hormone-free MS medium or by adding a small amount of BAP (0.2 mg/l). The latter hormonal formula provides an intense multiple shooting at *C. balsamita* too, and this fact can be of great use in the micropropagation of this species.

Using young axillary shoots to initiate the 'in vitro' culture at *C. balsamita,* multiplying the rosette-shaped shoots obtained on some hormonal formulae (as the above-mentioned formula) and their rooting on the hormone-free MS medium, especially on MS medium supplemented with 2 mg/l IAA and NAA or 1.0 mg/l IBA, gave vigorous neoplantlets. Root-striking efficiecy is very good. Neoplantlets adaptation to the septic environment was successfully accomplished and with insignificant biological material losses, in a hydroponic system, within 10 to 14 days. Accommodation system is more efficient at temperatures below 20°C. Two weeks after neoplantlets adaptation the regenerants obtained can be grown in field. For their survival in high percentage it is recomend to be taken out in field at the beginning of spring and to be watered periodically during 10 days of replanting. During the consecutive year to the transplantation the regenerants remain in the rosette stage, whilst the floriferous stems develop the next year.The regenerants are in bloom in August and September in the climatic conditions of Piatra Neamt.

CONCLUSIONS

The investigations dedicated to researching the 'in vitro' morphogenetic reaction of the *Chrysanthemum balsamita L.* species led to the following conclusions:

- 'In vitro' cultures initiation at this species can be accomplished using young axillary shoots as explants, harvested in August and September from the upper part of the stem and as nutritive mediums, the Murashige-Skoog hormone-free medium or the MS medium supplemented with small amounts of BAP (0.2 to 1.0 mg/l). These mediums give either neoplantlets or multiple rosette-shaped shoots.
- Inoculating rosette-shaped shoots on MS medium supplemented with BAP, or with BAP and IAA, or BAP and GA or BAP and zeatine provided a small-size compact callus at the contact point with the nutritive medium, that produced a gret number of shoots united at their base. Repeated subcultivation on mediums enriched in hormones favoured a phenomenon of hyperhydration.
- Cultivating rosette-shaped shoots on MS medium supplemented with 2 mg/l 2,4-D generated a compact rugged cream-greenish callus with a low capacity of cell multiplication. This hormonal formula provided callus too from fragments of leaves and roots, the callus being friable, cream-greenish or cream-coloured and with a low proliferation speed. The fragments of leaves produced callus even on MS medium supplemented with BAP and 2,4-D. Regardless its provenience, the callus did not prove having organogenetic capacity and degenerated in time.
- In order to obtain neoplantlets one recommends shoot multiplying on MS medium supplemented with 0.2 mg/l BAP and their transfer on MS medium supplemented with 2 mg/l IAA, NAA or 1 mg/l IBA in order to strike roots. Neoplantlets can easily adapt to septic environment in a hydroponic system and two weeks after accommodation they can be planted in field. During the first year after their transplantation in field, the regenerants develop a rosette of leaves, and the next year the floriferous stems are formed.

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Tabel 1 – The morphogenetic reaction of some *Chrysanthemum balsamita* L explants in 'in vitro' cultures

Var.	The explant	Hormonal formula	Growth regulators (mg/l)								The morphogenetic reaction and proliferation speed
			BAP	G	IAA	IBA	Kin	NAA	ZT	2.4 - D	
1	Shoots (rosette-shaped)	A	-	-	2,0	-	-	-	-	-	Neoplantlets (++++) with very well developed leaves and roots (+++)
2	"	B	0,2-2,0	-	-	-	-	-	-	-	Callus (+), compact , turning green when it getts in contact with the medium, that provides shoots shaped as multiple rosettes grown together (++++)
3	"	BA	1,0	-	0,5	-	-	-	-	-	Callus (+), compact, greenish, multiple rosettes grown together (++)
4	"	BN	1,0	-	-	-	-	0,5	-	-	Callus (+) compact, green when it contacts the medium, that develops multiple shoot rosettes (++)
5	"	BZ	1,0	-	-	-	-	-	0,5	-	Compact callus (+), like a spur, providing multiple rosette shoots (++)
6	"	GA	-	0,5	1,0	-	-	-	-	-	Neoplantlets (++), multiple shooting (+), root genesis (++)
7	"	GN	-	0,5	-	-	-	0,5	-	-	Neoplantlets (++); root genesis (+++) more intense than on GA
8	"	IB	-	-	-	1.0-2.0	-	-	-	-	Sporadically compact callus (+),cream - brownish; vigorous neoplantlets (+++) with very strong roots (++), multiple shooting (+)
9	"	KN	-	-	-	-	1.0	0,5	-	-	Neoplantlets (+++) and long, branchless roots (+++) and short narrow leaves
10	"	N	-	-	-	-	-	1,0-2,0	-	-	Compact light green callus (+) and intense root genesis (+++) at 1 mg/l NAA; neoplantlets with numerous and well-developed roots (++++)
11	"	D	-	-	-	-	-	-	-	2,0	Compact rugged cream-greenish callus when it getts in contact with the medium (+); strongly inhibited caulogenesis

12	Leaves	A	-	-	2,0	-	-	-	-	-	Explants grow evidently in sizes and frequently generate roots (++) at the end of the petiole
13	"	BD	1,0	-	-	-	-	-	-	0,5	Leaves thicken and on some parts of their lamina and petiole produce compact, light green callus (+)
14	"	BN	1,0		-	-	-	0,5	-	-	Roots (++) generated especially by the petiole
15	"	D	-	-	-	-		-	-	2,0	Friable or semicompact, cream or cream-redish callus (+)
16	"	IB	-	-	-	2.0	-	-	-	-	Compact callus (+) on some parts of the petiole; roots (+++) at the end of the petiole; fine roots in bunches (++) on some parts of the leaf stalk; leaf limb reactionless
17	Roots	A	-	-	2,0	-	-	-	-	-	Friable, cream-greenish callus (++) on its entire surface, or shaped as glomerules; sometimes fine roots (+) in bunches
18	"	D	-	-	-	-	-	-	-	2,0	Friable callus (++), cream-greenish on the entire length of the root fragments
19	"	IB	-	-	-	2.0	-	-	-	-	Compact, brown-greenish, filamentous callus (++)
20	Leaf callus obtained on D	B	1,0	-	-	-	-	-	-	-	Cream-greenish-brown callus (++), no organogenetic capacity
21	Stem callus on BN	B	1,0	-	-	-	-	-	-	-	Callus becomes caulogenetic (+), sporadically generating shoots

* BAP= benzyl-amino-purine; IAA= indolil-acetic acid; IBA= indolil-butiric acid; Kin= kinetine; NAA= naftil-acetic acid; G=giberelic acid; ZT= zeatine; 2,4-D= acid 2,4-D