COMPARATIVE STUDY ON THE REGENERATIVE AND ORGANOGENIC CAPACITY OF ECHINOPSIS (ZUCC.) CHAMAECEREUS F. LUTEA EXPLANTS, IN THE PRESENCE OF AN AUXIN IN THE CULTURE MEDIUM, NAMELY 3-INDOLYLBUTYRIC ACID (AIB) AND 2,4-DICHLOROPHENOXYACETIC ACID (2, 4D)

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RESEARCH ARTICLE

Abstract

Cactus with yellow skin, Echinopsis chamaecereus f. lutea, is part of a cactus chlorophyll - deficient, which occur spontaneously in culture because of mutation, they are unable to synthesize chlorophyll only survive if they are grafted.

In order to establish a culture in vitro of Echinopsis chamaecereus f. lutea, we have taken explants represented by seedlings from mother plants grown in the greenhouse. Inoculation of explants I did it on a culture medium consisting of macro Murashige-Skoog EDTA and Fe (1962), micronutrients Heller (1953), supplementation with medium supplemented with 3 indolylbutyric acid (AIB) in different concentrations, respectively: V_1 - 1 mg/l AIB; V_2 – 1.5 mg/l AIB; V_3 -2 mg/l AIB and V_4 medium supplemented with 2.5 mg/l 2.4 D (dichlorophenoxyacetic acid).

After 90 days of in vitro culture, it was found that on the Echinopsis chamaecereus f. lutea cactus, the presence of an auxin in the culture medium does not favor rhizogenesis. Explants grown on culture medium lacking growth regulators (V_0) were the only ones that generated both new stems and callus, while in vitro cultures grown on culture medium enhanced with 2 mg/l AIB (V_3) both the average number of calluses/variant as well as their average diameter equaled the control. In the current experiment, the beneficial effect due to the presence in the culture medium of 2.5 mg/l of 2,4-D (V_4) on the generation of callus was demonstrated, with an increase of 66.66%, and in what regarding the average diameter, this parameter marked an increase of 140% compared to the values of the control group V_0 (medium without growth regulators).

Keywords: cactus vitro cultures, 3-indolylbutyric acid (AIB), 2,4-dichlorophenoxyacetic acid (2,4-D), the callus, the newly formed stems.

INTRODUCTION

Regenerative and organogenic processes in in vitro cultures are greatly influenced by the presence in the culture medium of growth regulators, organic compounds with phytohormonal mimetic effects that play a primary role in the multiplication, growth and differentiation of "in vitro" culture explants, regardless of their type. Used in concentrations of 1 mM - 1.5 mM, or in even higher dilutions (in relation to the way of organization and endogenous content of hormones), growth regulators stimulate or inhibit the multiplication of explants, influencing the evolution of morphogenesis phenomena, depending on their nature, of the phytoinocula, their stage of development, etc. (Cachiţă, 1987; Cachiţă et al., 2004). According to Cachiţă et al. (2004) the presence of auxins in the culture medium stimulates cell division, they are involved in the phenomenon of apical dominance and have a rhizogenic action, inhibiting the formation of somatic embryos.

The ontogenetic development of plants is influenced by both endogenous and exogenous factors with more or less specific action. Plant hormones or growth regulators are organic compounds that stimulate or inhibit growth and morphogenesis, respectively regulate different physiological processes in plant tissues and organs (Davies, 2004).

Synthetic auxins include both 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-indolylbutyric acid (AIB), photohormones frequently used in tissue cultures with high efficiency in callus formation and rhizogenesis. It is known that 2,4-dichlorophenoxyacetic acid (2,4-D) added to the culture medium, in different concentrations, from 1 mg/l to 10 mg/l, stimulates the generation of callus at the explant level and plays an important role in increasing cellular metabolism. The formed callus explant can be detached, cut and then

transferred to fresh culture medium to obtain seedlings (Sandra Aparecida et al., 1996).

3 indolylbutyric acid (AIB) is a synthetic auxin, but it seems that it can be found in nature, but only in some plant species (according to Moore, Cachiţă et al., 2004). Auxins are commonly used in tissue culture to stimulate the rooting process.

Echinopsis chamaecereus f. lutea, is a chlorophyll-deficient cactus with a yellow epidermis (Copacescu, 2001), unable to synthesize chlorophyll due to the small number of chloroplasts, approximately 1/3 of the total plastids (Shemorakov, 2003). Russian researchers have shown particular interest in chlorophyll-deficient cacti species and thus have been classified according to the color of the epidermis (Shemorakov, 2003), according to these classifications, Echinopsis chamaecereus f. lutea is part of the monocolor group.

The pigmentation process is determined by the spontaneous appearance of mutations in cultures (Shemorakov, 2001) largely influenced by temperature and light. According to Skoulkin (2000), plants kept at lower than optimal temperatures or in the shade, develop such mutations very rarely, if at all. Through generative reproduction due to reversible mutations during meiosis (Shemorakov, 2003) there are minimal chances for these plants to retain their color (Kornilova, 2008), thus it was concluded that plants can only retain their color through in vitro cloning.

This fact has driven the search for new propagation technologies aimed at maintaining the specific characteristics of chlorophyll-deficient cacti, also fast, efficient and economical (Son, 2000, Lee et al., 2003).

The current experiment aims to study how the auxins 2,4-D (dichlorophenoxyacetic acid) and AIB (3-indolylbutyric acid) added to the culture medium act on the organogenic and regenerative capacity in explants of the chlorophyll-deficient cactus *Echinopsis chamaecereus f. lutea* . The variants we worked with were: V_0 - culture medium without growth regulators and media supplemented with auxins, as follows: V_1 - medium supplemented with 1 mg/l AIB; V_2 – 1.5 mg/l AIB; V_3 -2 mg/l AIB and V_4 - 2.5 mg/l 2.4 D.

MATERIAL AND METHOD

The biological material used in our experiments consisted of regenerated seedlings on *Echinopsis chamaecereus f. lutea* (fig. 1). The explants were about 1 cm long, 0,5 cm thick and a diameter of 0.5-1.5 cm, depending on the area from which they were harvested (fig. 2).

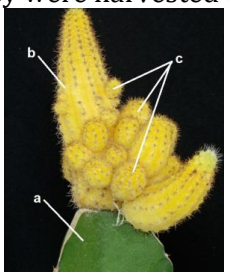

Fig.1.Young plant of *Echinopsis chamaecereus f. lutea*, (where: a-rootstocks; b-main stem; c-newly formed buds)

The vegetal material, seedlings of *Echinopsis chamaecereus f. lutea*, was asepsis by immersion, for one minute, in 96º ethyl alcohol, followed by its coating with 0.8% sodium hypochlorite solution, mixed with water in relation to 1: 2; To the disinfectant solution, three drops of Tween 20 are added (as a surfactant) (Cachiţă et al., 2004).

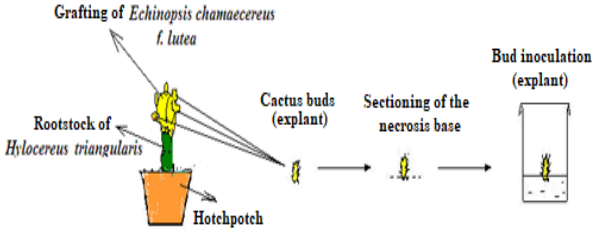

Fig.2. Schematic representation of the method of obtaining the explants of *Echinopsis chamaecereus f. lutea*, to be inoculated on aseptic media.

During the asepticization the vegetative material was stirred continuously (Cachiţă et al., 2004). After 20 minutes, the disinfectant was removed and the plant material was washed with sterile distilled water, making five consecutive rinses, five minutes each. Then, the plant material was deposited in aseptic conditions, in the hood with horizontal laminar flow, of sterile air, in operation, on the filter paper rounds sterilized in the oven, introduced in aseptic Petri dishes. Subsequently, the necrotic parts of the future inocula were removed.

Culture medium used for growth explants consisted of: macro Murashige-Skoog EDTA and Fe (1962), Heller microelements (1953), mineral mixture to which was added vitamins: pyridoxine HCl, thiamine HCl and nicotinic acid (containing 1 mg/l each), m-Inositol - 100 mg/l, sucrose - 20 g/l and agar 7 g/l the pH of the medium wasadjusted to 5,8, the first toautoclaving. In the basal medium (MB-V0) we added auxins, respectively 1 mg/l AIB (variant V1), 1.5 mg/l AIB (variant V2), 2

mg/l AIB (variant V3) and 2.5 mg/l l 2.4 D (variant V4).

Culture medium thus obtained was placed in a glass vial with a capacity of 15 ml (each container was placed 5 ml of medium). Medium vials were sterilized by autoclaving for 30 minutes at a temperature of 121°C. After cooling media proceeded to inoculate explants, aseptic room operation performed in a laminar flow hood with sterile air. To obstruction fitoinoculi containers we used polyethylene, immobilized with elastic. Containers were inoculated transferred to room for growth, under the following conditions: temperature ranged from 24ºC in the range of light and 20°C during the phase of darkness and light was the regime fotoperiodic 16 hours with light/ 4h, lighting achieving cultures with the white light emitted by fluorescent lamps, the intensity of 1700 lux.

Explants and explants reaction progress was monitored for 90 days. In this time periodwere conduct periodic observations and reading severy 30 days. Values thus obtained in the control group (V_0, the explants grow in basic medium, without growth regulators) were considered he reference as 100% to these all the other recorded values are related.

RESULTS AND DISCUSSIONS

Due to the small amount of chlorophyll pigments, *Echinopsis chamaecereus f. lutea*, cactus with a yellow epidermis, belongs to the category of chlorophyll-deficient cacti grafted on *Hylocereus triangularis* rootstock, which is due to a less developed root system.

This experiment lasted 90 days, during which periodical observations and recordings were made - at 60 and 90 days - of the monitored biometric parameters, the recorded values being related to those of the control group V_0 (the environment without growth regulators) which were considered to represent 100%. The results obtained, as well as their statistical significance, were centralized in table 1, while the graphic representations are illustrated by figures 3 and 4 and accompany the related discussions at 90 days of the experiment.

5 days after inoculation, we checked the percentage of infections, which was between 7 and 11% of the total containers inoculated with Echinopsis chamaecereus f. lutea explants, both on the basic medium without growth regulators (V_0), and and in the case of the other experimental variants (V_1 – V_3 and V_4) in which the aseptic substrate consisted of basic medium plus the growth regulator AIB, added in different concentrations (1 mg/l AIB – V_1; 1.5 mg/l AIB – V_2 or 2 mg/l AIB – V_3 and 2.5 mg/l 2,4-D - V_4). After 90 days of in vitro culture, the percentage of survival at the level of the four experimental variants was between 48-52% for variants V_0 - V_3 and 52-56% of the inocula for variant V4, a fact due to the necrosis that led to the death of the explants. According to (J van Staden and col, 2006) the genetic and morphological anomalies that appear in in vitro culture are most likely the result of oxidative stress induced either by the material used to obtain the inocula or by the presence of phytohormones in the culture media.

Comparing the results obtained on this date, it is found that the average length of the main stem both at the level of the phytoinocula of the variant V_1 (medium supplemented with 2.5 mg/l 2,4-D) and V_2 (medium supplemented with 2 mg/l BA) was located 0.3 cm (Fig. 4A) above the value of the same parameter recorded in the control group V_0 (medium without growth regulators), which represented an increase of 18.75% (Fig. 5A). Values that, statistically, are considered to be distinctly significant (Table 1).

It is noted that the addition of BA in the culture medium exerted a stimulating effect on the caulogenesis of vitro cultures of *Opuntia fragilis var. fragilis*, the average number of cauline neoformations exceeded the values of the similar biometric parameter in the control variant V_0 (medium without growth regulators) which with 1.4 new stems/variant is 35.72% below the value of this parameter in the explants of the V_2 variant (medium supplemented with 2 mg/l BA) with 3.7 kaolin neoformations/variant (Fig. 4B), while in the experimental variant V_1 (medium supplemented with 2.5 mg/l 2,4-D) an average number of 0.9 cauline neoformations/variant (Fig. 4B), thus registering a deficit of 35.72% (Fig. 5B), in relation to the values of the same parameter recorded in the control group V_0.

Culture medium of 2 mg/l BA (V_2) also stimulated the increase in the length of the newly formed stems up to an average value of this parameter of 6.5 cm (Fig. 3C), recording an increase of 91.17 (Fig. 4C), These results are considered, from the point of view of statistically, as being very significant (Table 1).

Table 1. The results of the biometric evaluations of the in vitro seedlings from 90 days after the inoculation of the explants on basic aseptic media (V_0) with the addition of 2.5 mg/l 2.4 D (V_1) and 2 mg/l BA (V_2)

Parameter / Alternative	The average length of the main stem	Standard deviation	Significance	Average number of newly formed stems +/- Standard deviation	Standard deviation	Significance	Average length of the largest newly formed stem+/-Standard deviation	Standard deviation	Significance	Average number of new roots +/- Standard deviation	Standard deviation	Significance	Average length of the largest newly formed root +/-Standard deviation	Standard deviation	Significance	Average number of calluses +/-Standard deviation	Standard deviation	Significance	The average diameter of calluses +/-Standard deviation	Standard deviation	Significance
90 days																					
V0	1,60±0,15	0,0211	***	1,40±0,45	0,2042	**	3,40±0,32	0,1053	***	1,70±0,29	0,0800	***	3,40±0,29	0,0863	***	0	0		0	0	
V1	1,90±0,40	0,1537	**	0,90±0,33	0,1079	*	3,20±0,84	0,7084	**	2,30±0,53	0,2763	**	4,70±0,75	0,5631	***	1,40±0,25	0,0632	**	2,40±0,30	0,0895	***
V2	1,90±0,36	0,1305	**	3,70±0,60	0,3505	***	6,50±0,61	0,3684	***	2,60±0,62	0,3790	**	3,40±0,68	0,4558	**	0	0		0	0	

Legend: *** very significant ** distinctly significant * significant NS insignificant

After 90 days from the initiation of the current experiment, the average basal diameter of the main stem in the case of the four experimental variants, in which the culture medium was supplemented with auxins, respectively: AIB added in different concentrations (1 mg/l AIB – V_1; 1.5 mg/l AIB – V_2 or 2 mg/l AIB – V_3) and 2.5 mg/l 2.4-D - V_4, is equal to, respectively, 1.2 cm (Fig. A), which represents an increase of 33.33% (Fig. A), compared to the values of the same parameter recorded in the control group V_0 (medium without growth regulators). Statistically, these values are considered to be distinctly significant (Table 1).

On this date, among the four variants of the culture medium studied, in this experiment it was found that only the explants belonging to the control lot V_0 (medium without growth regulators) generated cauline neoformations, in an average number of 0.3 buds/variant (Fig. B) with an average basal diameter of 0.4 cm (Fig. C).

Rhizogenesis did not occur, until this date, in any of the experimental variants studied. According to Corneanu, 2001, in most of the vitro-cultured species, the rhizogenesis process is easy on MS medium, supplemented with endogenous auxins, but species with a slow growth rate create particular problems for rooting.

Callus induction was observed in *Echinopsis chamaecereus f. lutea* explants inoculated and grown on culture medium lacking growth regulators (V_0) and on that supplemented with 2 mg/l AIB (V_3) as having the same values both in terms of concerns the average number of calluses/variant (0.3 calluses/variant) (Fig. D) as well as their average diameter - 0.5 cm (Fig. E). The results obtained by us are in agreement with those communicated by Corneanu et al., (1994), who reported that the explants of *Dilochothele longimamma*, cultured in vitro on Murashige-Skoog (1962) medium, without growth regulators, can generate both shoots and and callus.

The presence in the culture medium of 2.5 mg/l 2,4-D (V_4) stimulated callus formation to a greater extent; thus with an average number of 0.5 calluses/variant (Fig. D) at the level of explants grown on this substrate, an increase of 66.66% was recorded (Fig. B), while the average diameter of the callus (measured in the area the widest) was 1.2 cm (Fig. C), thus recording an increase of 140% (Fig. C), compared to the control group, results which, statistically, are interpreted to be significant (Table 1). These results are in agreement with those reported by Medeiros et al., (2006), who obtained massive callus formation in Notocactus magnificus vitrocultivated on medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid.

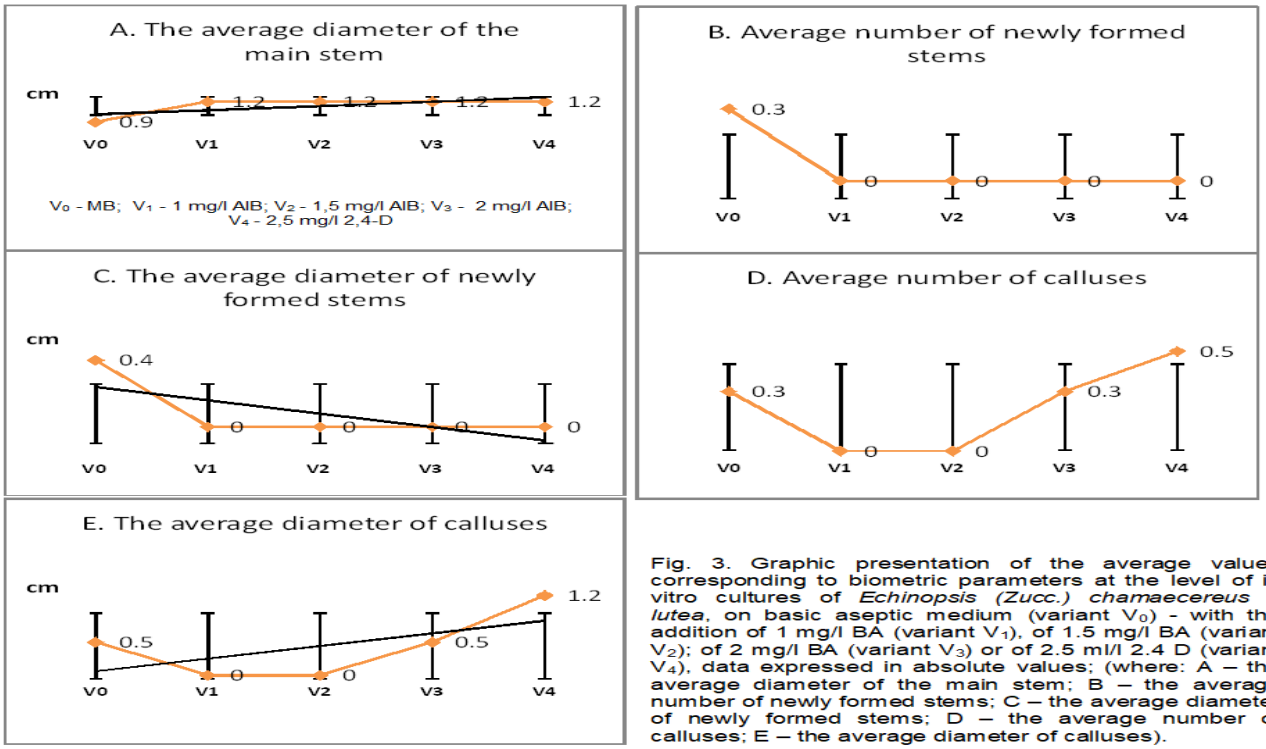

Fig. 3. Graphic presentation of the average values corresponding to biometric parameters at the level of in vitro cultures of *Echinopsis (Zucc.) chamaecereus f. lutea*, on basic aseptic medium (variant V_0) - with the addition of 1 mg/l BA (variant V_1), of 1.5 mg/l BA (variant V_2); of 2 mg/l BA (variant V_3) or of 2.5 ml/l 2.4 D (variant V_4), data expressed in absolute values; (where: A – the average diameter of the main stem; B – the average number of newly formed stems; C – the average diameter of newly formed stems; D – the average number of calluses; E – the average diameter of calluses).

By analyzing the images in the figure, it can be observed that, at the level of the explants of *Echinopsis chamaecereus f. lutea* belonging to the V_0 variant (medium without growth regulators), the areoles and spines are normally developed, characteristic of the species, it should be noted that, on the surface of the explant, in addition to the existence of necrotic areas, there are also surfaces colored in shades of green, a fact also observed at the level of kaulin neoformations. The callus formed is located on the surface of the main and newly formed stems and has a yellowish-cream color.

At the level of explants inoculated and grown on medium supplemented with AIB auxin (fig.), it can be observed that the explants of the three experimental variants (V_1, V_2 and V_3) kept their yellow color, areolae and spines characteristic of the species. Explants inoculated and grown on medium supplemented with 2.5 mg/l 2,4-D (V_4) developed a dense optical callus, compact, with a velvety appearance, located at the base or on the surface of the explant, almost entirely covering the nutrient substrate ; it is differently colored, from white, yellow to pink, or even shades of orange (Fig. 61B, C and D), a fact also noted in the case of vitro culture of *Echinocactus mihanovichi,* (Vidican, 2012) on the same type of nutrient substrate.

Based on the analysis of the data obtained in the present experiment, it can be noted that the results obtained by "in vitro" cultivation of the species *Echinopsis chamaecereus f. lutea* on a basic culture medium improved with an auxin, respectively AIB in different concentrations (V_1 - 1 mg /l AIB; V_2 - 1.5 mg/l AIB; V_3 - 2 mg/l AIB) and 2.5 mg/l 2,4-D (V_4) are not satisfactory in relation to the goal pursued by us, thus , in terms of regenerative and organogenic capacity, the most beneficial proved to be the environment without growth regulators (V_0), the explants inoculated and grown on this nutrient substrate generated new stems and callus, a phenomenon manifested since the first 60 days of experiment. On the culture medium supplemented with 3-indolylbutyric acid (AIB), in different concentrations, no response was received regarding the organogenic potential of the explants, with the exception of variant V_3 (medium supplemented with 2 mg/l AIB) in which, after 90 days of in vitro culture, the phenomenon of callus induction was potentiated, a fact noted also in explants of *Echinopsis chamaecereus f. lutea*, grown on culture medium supplemented with 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4- D) (V_4), which stood out both for the highest number of calluses/variant, with an increase of 66.66%, and their sizes, this parameter marking an increase of 140%.

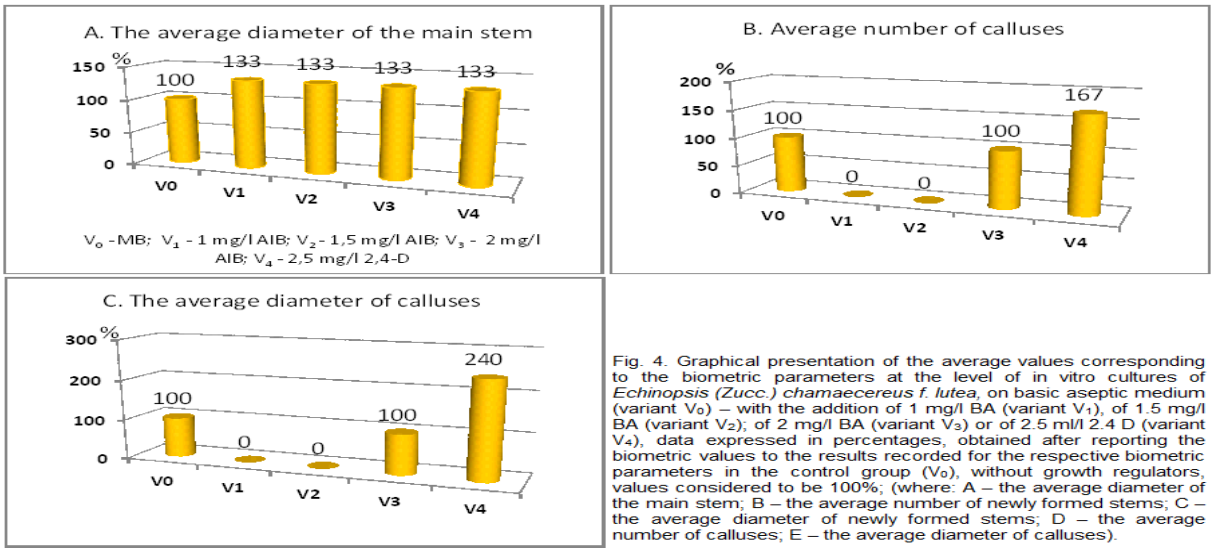

Fig. 4. Graphical presentation of the average values corresponding to the biometric parameters at the level of in vitro cultures of *Echinopsis (Zucc.) chamaecereus f. lutea*, on basic aseptic medium (variant V_0) – with the addition of 1 mg/l BA (variant V_1), of 1.5 mg/l BA (variant V_2); of 2 mg/l BA (variant V_3) or of 2.5 ml/l 2.4 D (variant V_4), data expressed in percentages, obtained after reporting the biometric values to the results recorded for the respective biometric parameters in the control group (V_0), without growth regulators, values considered to be 100%; (where: A – the average diameter of the main stem; B – the average number of newly formed stems; C – the average diameter of newly formed stems; D – the average number of calluses; E – the average diameter of calluses).

CONCLUSIONS

From the data monitored and examined for 90 days, we found the particularly favorable effect of benzyladenine (BA) on caulogenesis in vitro cultures of *Opuntia fragilis var. fragile*. The explants inoculated and grown on culture medium supplemented with 2 mg/l BA (V_2), were distinguished by the most new strains/variant, respectively by 164.28% above the values recorded in the control lot compared to the phytoinocula belonging to the V_1 variant (culture medium supplemented with 2.5 mg/l 2,4-D) where an increase of 64.28% was marked.

The average length of the largest newly formed shoot is also found in the explants grown on culture medium supplemented with 2 mg/l BA (V_2) which, with 6.5 cm, registered an increase of 91.17% compared to the control group without of growth regulators (V_0) compared to 3.2 cm the value of the same parameter in the phytoinocula belonging to the V_1 variant (culture medium supplemented with 2.5 mg/l 2,4-D), which represents a deficit of 5.89% .

Rhizogenesis is a phenomenon manifested in all the experimental variants studied, thus with an average number of 2.3 roots/variant, the explants grown on culture medium supplemented with 2.5 mg/l 2,4-D (V_1) exceeded the control by 35.29% compared to 2.6 roots/variant in the explants on culture medium supplemented with 2 mg/l BA (V_2) which represents an increase of 52.94%. The presence of auxin in the culture medium positively influenced the length of the largest root, so in the explants of variant V1 (culture medium supplemented with 2.5 mg/l 2,4-D) it was 4.7 cm, an increase of 38 .23%, compared to 3.4 cm in V_2 (culture medium supplemented with 2 mg/l BA) which equaled the control.

The presence in the culture medium of 2,4-dichlorophenoxyacetic acid (2,4-D) had a stimulating effect on callusogenesis, only at the level of the explants grown on this medium (V_1) 1.4 calluses/variant were generated, which grew until they reached an average diameter of 2.4 cm.

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