# COMPARATIVE RESEARCH ON THE REGENERATION AND ORGANOGENIC CAPACITY OF IN VITRO CULTURES OF *Opuntia fragilis var. fragilis*, IN THE PRESENCE IN THE CULTURE MEDIUM OF AUXIN - DICHLORPHENOXYACETIC ACID - 2.4 D (2.5 mg/l) AND OF CYTOQUININE - BENZYLADENINE - BA (2 mg/l)

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RESEARCH ARTICLE

**Abstract**

*Among the 130 genera of the Cactaceae family, the genus Opuntia is one of the most studied in the world (Griffith, 2001a, b, 2004; Pinkava, 2002). The species of this genus of cactus are, from an economic point of view, the most widespread and important of the Cactaceae family, the fruits and plant mass are edible (Nobel, 2002; Nobel et al., 2002), they are extremely efficient in transforming water into biomass (Kluge et al., 1978, Griffith, 2004a), being also recognized as an indicator of some noxes (Nobel, 1994). Due to their great adaptability to harsh environmental conditions, cacti belonging to the genus Opuntia are found in arid and semi-arid areas used to combat desertification (Le Houérou, 2000; Juárez et al., 2002). There is evidence that Opuntia plants (fig.1) have been used by humans for at least 9000 years (Kiesling, 1998).*

*For the initiation of in vitro cultures of Opuntia fragilis var. fragilis we sampled stem fragments sectioned into portions approximately 1 cm long and 0.5 cm thick with at least 2-3 areoles. The portions thus taken were deposited on the sterilized culture medium consisting of macroelements and Fe EDTA Murashige-Skoog (1962) microelements Heller (1953), supplemented with 2.5 mg/l 2.4-dichlorophenoxyacetic acid ($V_1$) and 2 mg/l benzyladenine ($V_2$).*

*The evolution of the in vitro cultures was monitored for 90 days. Their response was different, the presence of auxin, respectively, 2.5 mg/l 2.4-D ($V_1$) in the culture medium determined the formation of new roots 2.3/variant, respectively an increase of 35.29% with a length average of the largest generated root of 4.7 cm, an increase of 38.23%, also being the only variant on which callus formation was observed, with 1.4 calluses/variant and an average diameter of 2, 4 cm. The favorable effect of cytokinin was noted by the formation of new strains in the medium supplemented with 2 mg/l BA ($V_2$) where we recorded 3.7 newly formed strains/variant, so an increase of 64.28% with an average length of 6.5 cm, respectively an increase of 91.17, all data were reported to the control group lacking growth regulators ($V_0$).*

**Keywords**: Rhizogenesis, caulogenesis, callus, phytoinoculation.
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## INTRODUCTION

Auxins, along with cytokinins, belong to the category of growth regulators or phytoregulators, organic compounds with mimetic phytohormonal effects (Cachiţă, 1987; Cachiţă et al., 2004).

Fig.1. Image representing the cactus *Opuntia fragilis var. fragilis*, where: a-stems; b-flower; c-fruit; d-areoles; e-thorns.

Auxins have a rhizogenic action (Cachiţă et al., 2004), according to Sandra Aparecida et al., 1996, the addition of dichlorophenoxyacetic acid (2,4-D) to the culture medium is highly effective in rhizogenesis and callus formation, which subsequently it can be detached, cut and then transferred to a fresh culture medium to obtain seedlings, but in higher concentrations it becomes toxic.

The addition of benzyladenine (BA) boosts the formation of buds at the inoculum level, from which new stems will be generated (Mauseth, 1976), exerting an antagonistic effect on auxins, inhibiting rhizogenesis (Cachiţă et al., 2004). In in vitro cactus cultures, it is considered that, in order to multiply plant material, the most effective growth regulator to generate new stems is BA (Escobar et al, 1986).

The aim of this research is to study how the presence in the culture medium of an amount of 2.5 mg/l 2.4 dichlorophenoxyacetic acid (2.4 D) and 2 mg/l benzyladenine (BA) influences the regenerative capacity and organogen of vitroplants of *Opuntia fragilis var. fragilis*, knowing that it is believed that cacti invariably induce organogenesis processes when they are grown on media supplemented with growth regulators (Copăceacu, 2001).

## MATERIAL AND METHOD

To initiate *in vitro* cultures of *Opuntia fragilis* var *fragilis* i keep prelevet strains with mature areolas but with less thorns trainers, shorts and white. The material so obtained was secţiont transverse operation which resulted dished washers that were divided so that eventually fragments were inoculated following dimensions: about 1 cm long and 0,5 cm thick, yet have minimum 2-3 areola. After these operations we obtain the explants from mid dial and lateral (Fig. 2).

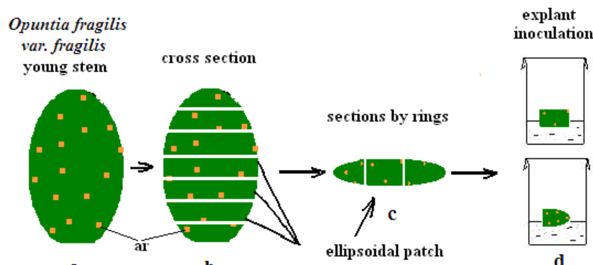


Fig.2. Schemetic representation of *Opuntia fragilis* var *fragilis* young stems (a, b), and how slicing it into rings ellipsoid (c) and lateral explants inoculated on media centers and aseptic (d), where: ar - areola.

Knowing that *in vitro* cultures of naturally occurring cacti - the areola - some long hairs and bristles, host parties for a variety of organisms (Garcia-Saucedo et al., 2005), sanitized of plant material was achieved by submersare for one minute at 96 º alcohol, followed by the coating process it with a solution of 0.8% sodium hypochlorite mixed with water in a ratio of 1:2, which were added three drops of Tween 20 as surfactant (Cachiţă et al., 2004). Sanitized lasted 20 minutes, during which the plant material was continuously stirred. After decanting disinfectant plant material was washed with sterile distilled water to remove chlorine, achieving five consecutive rinses, of five minutes each.

After sterilization, the plant material was deposited in Petri capsules on filter paper discs (previously sterilized in the oven) in a laminar flow hood, horizontal air sterile operation, followed by sizing operation and future inocula removal of necrotic parts thereof.

The culture medium used for the growth of explants consisted of: macro Murashige-Skoog EDTA and Fe (1962), trace elements Heller (1953), mineral mixture to which vitamins were added: 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 was adjusted to a value of 5.8, its first autoclaving. In the basic medium (MB) shown, we added 2.5 mg/l 2,4-D and 2 mg/l BA, obtaining the following variants: $V_0$ - control, medium without growth regulators and $V_1$ - supplemented medium with 2.5 mg/l 2,4-D and $V_2$ - medium supplemented with 2 mg/l BA.

The culture medium was placed in a glass vial with a capacity of 15 ml (each container was placed 5 ml of medium). Medium vials were sterilized for 30 minutes, by autoclaving 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 inocula were transferred to room for growth, under the following conditions: temperature ranged from 24ºC in peroada light and 20° during the phase of darkness and light was the regime fotoperiodic 16 hours lumină/24h, lighting cultures achieving is the white light emitted by fluorescent lamps, the intensity of 1700 lux.

Reaction and evolution of explants was monitored for 90 days. In this time period were conducted periodic observations and readings every 30 days. Values recorded biometric control group ($V_0$, fitoinoculi grown on basic medium, without growth regulators) were considered the reference as 100% being reported - every trait - all readings averaged every experimental variant part.

## RESULTS AND DISCUSSIONS

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 $V_0$.

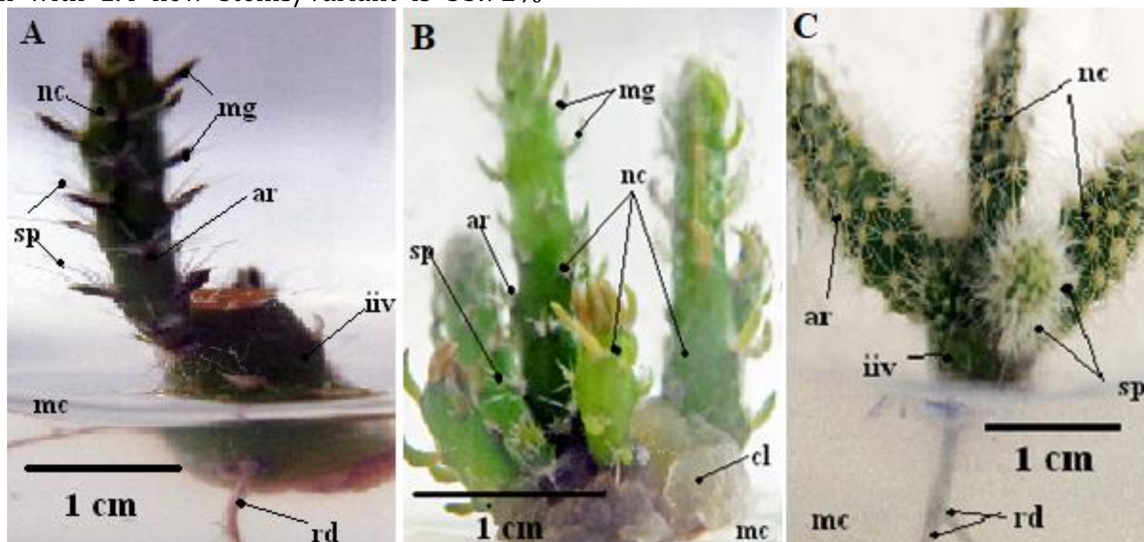


Fig.3. Inoculi de *Opuntia* (Tournef.) Mill. *fragilis* var. *fragilis,* la *90 de zile* de la inocularea explantului *„in vitro”,* unde: A-mediu de bază modificat de noi şi lipsit de regulatori de creştere ($V_0$); B-mediu de bază cu adaos de 2,5 mg/l 2,4-D ($V_1$); C-mediu de bază cu adaos de 2 mg/l BA ($V_2$); (iiv–inocul iniţial viabil; mc–mediu de cultură; nc-tulpini noi; rd-rădăcini; ar-areole; sp-spini; cl-calus; mg-muguraşi).

And regarding the average length of the largest newly formed stem, the addition of auxin, 2.5 mg/l 2,4-D ($V_1$) determined a minus of 0.2 cm (Fig. 4C) in relation to those 3.2 cm recorded in the control V0 (medium without growth regulators), which represents a deficit of 5.89% (Fig. 40C), while the presence in the 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. 4C), recording an increase of 91.17 (Fig. 5C), 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

Fig.4. The graphic presentation of the average values, in absolute values, corresponding to the biometric parameters at the level of vitro cultures of Opuntia (Tournef.) Mill. fragilis var. fragilis, on a base aseptic medium modified by us - (variant V0), with the addition of 2.5 mg/l 2,4-D (variant V1) and 2 mg/l BA (variant V2), data expressed in absolute values; (where: A–average length of the main stem; B–average number of new stems; C–average length of the largest new stem; D–average number of roots; E–average length of the largest root; F–number callus average; G-average callus diameter.

Following the results obtained, it can be said that the presence of cytokinin in the culture medium - in the case of this experiment of benzyladenine (BA) added in concentrations of 2 mg/l (V2) - a greater number of new strains/variant is also obtained, and the length of the shoots on the explant is considerably longer, results similar to those reported by Vidican et al., 2010, also, the results obtained allow us to consider that we are in agreement with the reports made by Khalafalla et al., 2007, which reached the conclusion that the most effective culture medium in terms of the number and length of shoots in vitro cultures of *Cactacaea*, is the medium supplemented with benzyladenine (BA), these parameters reaching the maximum value at a concentration of 5.0 mg/l BA. I also noted that the presence of BA in the culture medium represented a favorable stimulus that determined the swelling of 100% of the areoles, differentiating a variable number of buds from them, an aspect also noted by Juárezi et al., (2002).

The newly formed stems from the explants grown on mediums lacking growth regulators as well as those supplemented with BA cytokinin or 2,4 D auxin maintain their intense green color, and the areoles are well developed, the long, white spikes that keep the same fluffy appearance (Fig.3). However, it was found that, in the control group without growth regulators ($V_0$) and in the one supplemented with 2 mg/l BA ($V_2$), the stems have a much higher number of thorns (Fig.3) compared to the stems regenerated from the explants cultivated on medium with 2.5 mg/l 2,4-D ($V_1$) in which the thorns - modified leaves - were much rarer, but quite long, a fact that could be a consequence of the "defoliation" action that auxin 2,4-D - a herbicide with a defoliating effect - to have exercised it in this case (Fig. 3), also, the presence of a large number of buds can be found at their level. It should be noted that in the explants grown on medium supplemented with 2 mg/l BA ($V_2$), first-order ramifications were generated at the neostem level.

Regarding rhizogenesis, at this time it was noted that this phenomenon was manifested in all the experimental variants studied. The average number of roots generated at the level of explants grown on medium supplemented with 2.5 mg/l 2,4-D ($V_1$), was 0.6 roots/variant above the value of the same parameter recorded in the control

group $V_0$ (medium without growth regulators) Fig. 4D), which represents an increase of 35.29% (Fig. 5D), while at the level of explants of *Opuntia fragilis var. fragilis* grown on medium supplemented with 2 mg/l BA ($V_2$) was 2.6 roots/variant, respectively an increase of 52.94% (Fig.5D). These differences, from a statistical point of view, are considered to be distinctly significant (Table 1).

The presence of 2,4-D auxin in a concentration of 2.5 mg/l ($V_1$) in the culture medium also positively influenced the growth in length of the newly formed roots, thus the average length of the largest root - in absolute values - exceeded the value recorded by this parameter in the control group by 1.3 cm (Fig. 4E), marking an increase of 38.23% (Fig. 5E); these differences are considered, from a statistical point of view, to be very significant (Table 1). It is found that this parameter had an average absolute value of 3.4 cm in the case of explants of Opuntia fragilis var. fragilis inoculated and grown on culture media supplemented with 2.0 mg/l BA ($V_2$) so they managed to equal the control $V_0$ (Fig.4E).

In the phytoinoculi grown on culture medium supplemented with 2.0 mg/l BA ($V_2$) but also in the control sample $V_0$ (medium without growth regulators) (Fig.3), callus formation was not observed. In the explants of *Opuntia fragilis var. Fragile*. The presence in the acid culture medium of 2.5 mg/l 2,4-D ($V_1$) had a stimulatory effect on callusogenesis which was manifested only at the level of explants grown on this medium where 1.4 callus/variant were generated, a 140% increase compared to the control group $V_0$ (medium without growth regulators) (Fig. 4F); this growing until they reached an average diameter of 2.4 cm, respectively an increase of 240% (Fig. 5G). These results are considered, from a statistical point of view, to be very significant (Table 1).

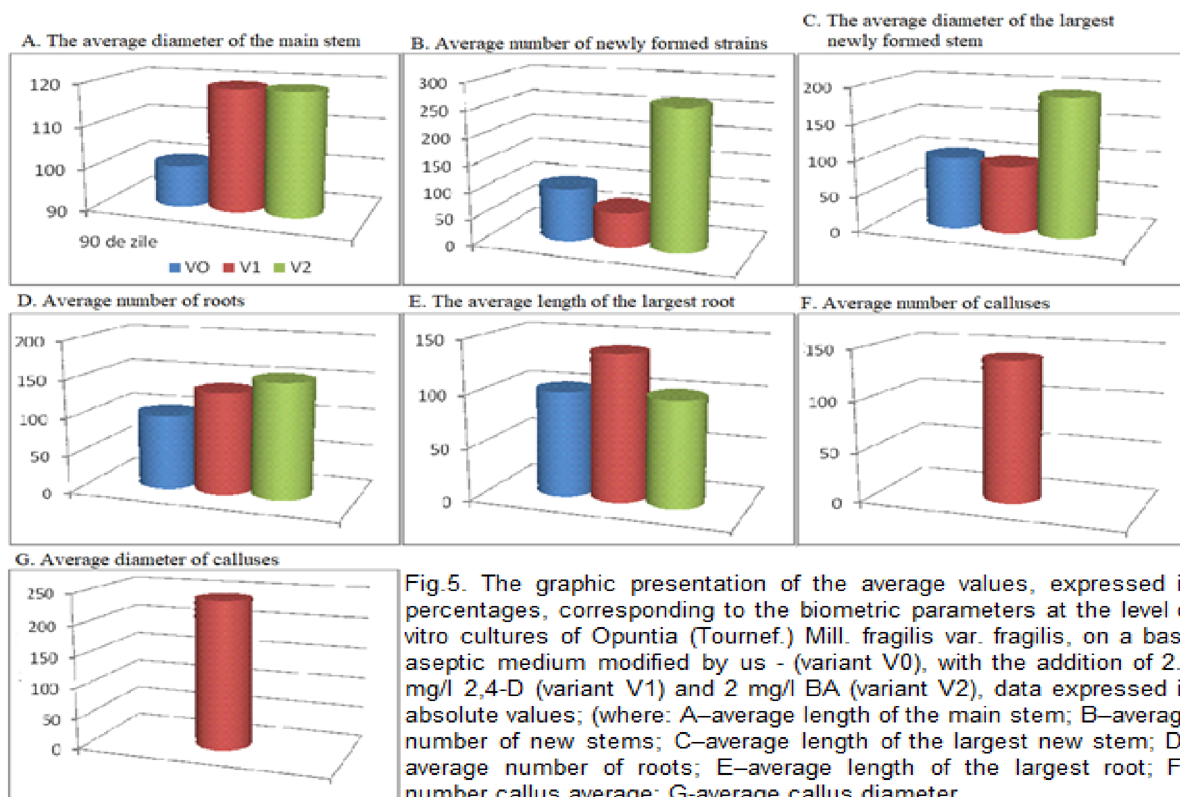


Fig.5. The graphic presentation of the average values, expressed in percentages, corresponding to the biometric parameters at the level of vitro cultures of Opuntia (Tournef.) Mill. fragilis var. fragilis, on a base aseptic medium modified by us - (variant V0), with the addition of 2.5 mg/l 2,4-D (variant V1) and 2 mg/l BA (variant V2), data expressed in absolute values; (where: A–average length of the main stem; B–average number of new stems; C–average length of the largest new stem; D–average number of roots; E–average length of the largest root; F–number callus average; G-average callus diameter.

In the case of our experiment, the callus generated at the explant level of *Opuntia fragilis var. fraglis* inoculated and grown on culture medium supplemented with 2.5 mg/l 2,4-D ($V_1$) either due to the abundance it covered the entire surface of the nutrient substrate (Fig. 41B), in which case it shows signs of early semiescence - fact highlighted by the cream color - it was located at the base of the neostems in the form of opalescent, friable, pale green tissue (Fig.3).

**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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