SUMMARY OF WORKS
2008

The problem of graft compatibility is still insufficient known , therefore is absolutely necessary to take account to this phenomenon in practices of grafting . Whereas, until the present, the methods for certain appreciation of compat ibility before the grafting process are not yet known, the affinity between partners rest to be appreciated only after grafting and a long "cohabitation". The long time, necessary for incompatibility symptoms appearance *in vivo* makes difficult the research es in this field. Many interpretation problems are due to the influence of many exogen factors on cell regeneration processes which evolves at the grafting area. All these inconvenient can be eliminates by using as biological material the *in vitro* grafted plants.

Generally, the intimate phenomenon related with transformation of definitive morphologically cells in meristematic cells *in vitro* are, in a great measure corresponding with the similar processes developed in plants in case of the usual vegetative multiplication.

It can appreciate that dedifferentiation cell explants, occurs in conditions of removal of tissues from the interdependency in which they gr ew until then, removing in the same time the entire complex of factors and stimulus which exercised a repression on the manifestation of the capacity of the cell to develop, in the integral organism structural context.

The problem seen through this prism leads us to conclude that, *in vitro*, cells can be dedifferentiate faster and easier than maintaining t heir place of origin. Thus using cell cultures and plant tissues as products of in vitro experimental biological material offers many advantages over the in vivo biological material. These include: minimizing the influence of environment on the material us ed, possibility of obtaining large numbers of individuals genotypic uniform in a small space, throughout the ye ar, acceleration of the growth and regeneration processes etc.

Particular interest in recent decades has given the possibility of in vitro cultivation in temperate tree species. Possibility of shoots by meristematic culture has led to the development of micropropagation techniques and their use as alternative to standard methods of propagation. On the other hand , many wood species were found to be unable to regenerate whole plants from plant tissue culture . For recalcitrant species, in order to obtain virus free plants, Murashige and his colleagues have developed a technique of grafting in vitro, named micrografting. This technique began to be increasingly used for various purposes micropropagation, production of pathogen free plants, and also in order to elucidate some issues of incompatibility phenomenon manifest in various fruit species.

One of the pioneers in implementing this technique in citrus was Navarro (1975) where good results have been obtained to other species *Citrus* spp., *Malus pumila* L., *Prunus armeniaca* L. , *Prunus amygdalus* L. (Yeomann, M. M., Kilpatric, D. C., Miedzybrodzka, M. B. ,Gould,A. R. 1978, Parkinson, M. , Yeomann, M. M. 198 2, Jeffree, C. E., Yeoman, M. M. 1983, Navarro, L. 1988) etc.

Parkinson and Yeoman made anatomical and morphological studies of in vitro micrografted plants and Cantor described morphological anatomical details (callus, and formation of vascular junctions) at *Vitis vinifera* L.

Later this technique was successfully applied to elucidate some classical problems of graft rootstock- scion incompatibility, polyphenolic substances and resins production (Joley et Opitz, 1971; AI-Barazi et Schwabe, 1982 ; Sheibani et Villiers, 1995, Jonard et al., 1988) or s or scion rejuvenation (Revilla et al.,1996; Estrada-Luna et al., 2002).

Micrografting technique involves the following steps: obtaining rootstocks which is done by aseptic germination of seeds, preparation of scions which are obtained by micropropagation techniques or somatic organogenesis, micrografted aseptically growing plants and plant acclimatization to *ex vitro* conditions of life.

For obtaining the rootstocks we used stratified seeds from *Pyrus sativa* and *Cydonia oblonga* and seeds from the following cultivars: Curé (compatible with both rootstocks) and Triumf compatible with *Pyrus sativa* but incompatible with *Cydonia oblonga*. We used also seeds from *Prunus armeniaca* and *Prunus cerasifera* as rootstocks and seeds from the apricot cultivar Goldrich.

In vivo seed germination

20 stratified seeds from each cultivar were sown in a mixture of peat and soil 1:1 and covered with plastic to maintain humidity. The experiment was repeated three times to provide statistical data. Observations were made at 30-60 days after sowing.

Seeds sterilization

Mature seeds were divided into two groups: intact and with removed pericarp. Both were presterilized with 70% ethanol for 45 sec. then washed with sterile distilled water. Intact seeds were treated with sulfuric acid in concentrations of 1, 5, 10 and 36 N for 30 minutes. Subsequently all the seeds were sterilized with sodium hypochlorite 5% (v / v) for 15 min. and washed three times with sterile distilled water.

In vitro seed germination

For germination seeds were placed in sterile Erlenmeyer flasks both filter paper soaked with sterile distilled water, as well as Murashige and Skoog media, 30 gl -1 sucrose, 6.3 gl-1 agar (Merck), pH 5.7, sterilized previously by autoclaving for 15 minutes at 121 C.

To test the effect of phytohormones on seed germination were determined following hormonal balance:

V1-MS +1mg/l2,4D+1mg/l K
V2-MS +1mg/l2,4D+1mg/l BAP
V3-MS +1mg/l2,4D+5mg/l AIA
V4-MS +1mg/l2,4D+1mg/l K+5mg/l AIA
V5-MS without phytohormones

Light from a fluorescent tube located at a distance of 30 -35 cm from the shelf area with intensity of 40 µmol m fotofluxului -2s-1 with a photoperiod of 16 / 8. Observations were made periodically at 14 days of incubation.

Results

For most varieties the germination rate ranged between 12 and 54% after 60 days of sowing. At the same time it may be noted that in vitro seed s germination shell, even if their scarification were worse than in vivo variants. Germinated seeds made on sterile filter paper had a higher germination rate than if they are placed on medium without hormones. In this case the stagnation of the germination process can be attributed to seed

hydration. In vivo seed germination started 12 days after sowing and 30 days after germination has been achieved 2/3 of total germinated seeds.

In experiments in vitro germination process went quickly, the apricot germination began at 3 days after placing seeds on culture medium by the appearance of rootlet and 7th day was found strains appearance.

. Table 1

Seedlings obtained by morphometric values of in vivo and in vitro germination of seeds of different species of trees

Explant	Culture media	Germination percent (%)	Shoot's length (mm)	rootlet length (mm)
Pyrus sativa seeds	mixture of peat and soil in vivo	54	38,42	39,45
Curé seeds		39	28,28	34,65
Triumf seeds		42	31,84	43,62
Cydonia oblonga seeds		52	41,39	51,13
Prunus armeniaca seeds		12	11.3	52,7
Paddy seeds				
Pyrus sativa seeds	Filter paper	38	46,7	37,8
	MS without phytohormones	11	44,2	33,6
Curé seeds	Filter paper	21	31,6	28,9
	MS fara hormoni	9	21,5	24,6
Triumf seeds	Filter paper	34	21,7	19,5
	MS without phytohormones	21	27,9	21,4
Cydonia oblonga seeds	Filter paper	0	0,	0
	MS without phytohormones	0	0	0
Prunus armeniaca seeds	Filter paper	0	0	0
	MS without phytohormones	0	0	0
Husked seeds				
Pyrus sativa seeds	MS +1mg/l2,4D+1mg/l K	14	32,5	30,6
	MS +1mg/l2,4D+1mg/l BAP	32	36,7	33,4
	MS +1mg/l2,4D+5mg/l AIA	11	21,4	21,3
	MS +1mg/l2,4D+1mg/l K+5mg/l AIA	10	12,7	22,4
	MS without phytohormones	5	11,4	16,8
Curé seeds	MS +1mg/l2,4D+1mg/l K	21	26,7	24,6
	MS +1mg/l2,4D+1mg/l BAP	36	28,4	31,6
	MS +1mg/l2,4D+5mg/l AIA	2	9,4	23,6
	MS +1mg/l2,4D+1mg/l K+5mg/l AIA	1	10,4	21,8
	MS without phytohormones	0	0	0
Triumf seeds	MS +1mg/l2,4D+1mg/l K	9	13,5	18,9
	MS +1mg/l2,4D+1mg/l BAP	12	18,4	48,9
	MS +1mg/l2,4D+5mg/l AIA	3	2,4	39,7
	MS +1mg/l2,4D+1mg/l K+5mg/l AIA	2	2,5	31,
	MS without phytohormones	0	0	0
Cydonia oblonga	MS +1mg/l2,4D+1mg/l K	0	0	0
seeds	MS +1mg/l2,4D+1mg/l BAP	7	2,7	11,4
	MS +1mg/l2,4D+5mg/l AIA	0	0	0
	MS +1mg/l2,4D+1mg/l K+5mg/l AIA	0	0	0
	MS without phytohormones	0	0	0
	MS +1mg/l2,4D+1mg/l K	11	11,4	58,9
Prunus armeniaca	MS +1mg/l2,4D+1mg/l BAP	42	14,7	68,4
seeds	MS +1mg/l2,4D+5mg/l AIA	17	10,6	52,4

	MS +1mg/l2,4D+1mg/l K+5mg/l AIA MS without phytohormones	12 5	9,3 9,8	38,9 28,4

Effect of growth regulators on the morphology of seedlings in vitro

Phytohormones add to the growing medium had different effects on both the process of germination and seedlings growth and development . As a first aspect can be seen that the addition of any hormone in the culture medium had a stimulating effect on germination, however, better results were found when adding c ytokine than auxin. The second cytokine added BAP was more pronounced stimulatory effect than ki netin. Also, the same culture medium supplemented with 2,4 D and BAP were recorded and most pronounced increases in seedlings. Indolil acetic acid was only stimulatory effect on root growth.

Given the results obtained in the first phase experiment to prepa re rootstocks, but also for growth and development of microgrfted plants it has been continue to use the version of MS medium supplemented with BAP .

Scion's preparation

As scions, in vitro propagated shoots were used. For this purpose was harvest apical meristem of active or physiological resting estate from which we obtained from meristem nodal cuttings were sterilized by immersion in 70% ethanol for a few seconds, then the solution of sodium hypochlorite 5% for 10, 15, 20 minutes. After sterilization cuttings were placed on MS culture medium with various hormonal balances and kept for a month. Of these cuttings were harvested micro-scions measuring 3-15 mm were used for micrografting

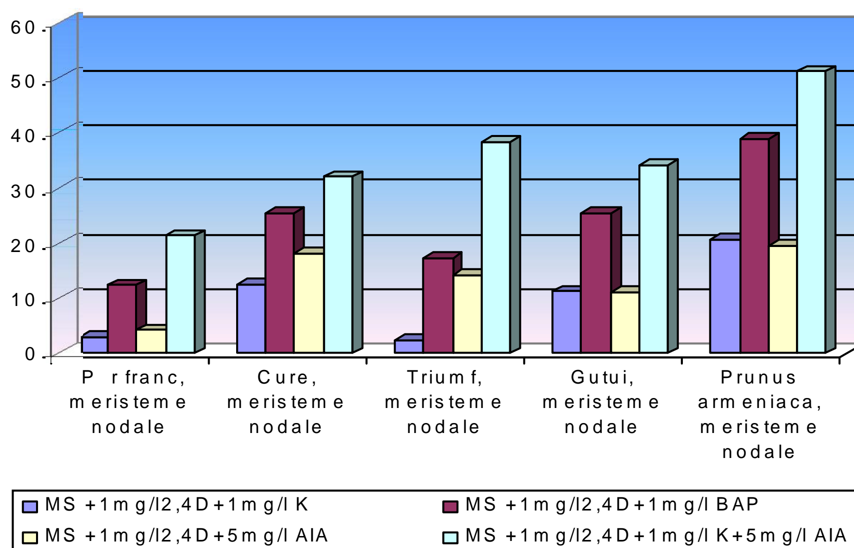


Fig 1. Variation in capacity for regeneration of shoo ts from nodal meristem of different tree species on different versions of MS medium

Cytokines are a limiting factor if the regeneration of shoots in woody plants. Among the studied cytokines highest rate of regeneration of shoots in vitro all the studied species showed a BAP, however, the combination of cytokines auxines and gave satisfactory results in *Prunus armeniaca*, *Cydonia oblonga* and pear variety Triumph.

The results obtained showed that MS medium supplemented cu1mg/l2, 4D 1 mg / l K 5 mg / l IBA has helped obtain shoots in all studied species. Satisfactory results were obtained from variety Cure, *Cydonia oblonga* and *Prunus armeniaca* 1mg/l2 supplied with 1mg/l2,4D+1mg/l BAP and media supplied with 1mg/l2,4D+1mg/l K and 1mg/l2,4D+5mg/l AIA does not favorite shoots induction.

Grafting procedure:

10 seedling rootstocks were decapitatedat about 25 to 40 mm height and 1.5 to 5.0 mm diameter at 50 day s*in vitro*. Grafting was done aseptically by inserting a piece (3 -15mm) of the scion. A downward central incision of 5-10 mm was made and the wedge of microscion was inserted gently and firmly into the vertical split on the decapitated seedling.

In vitro micrografts cultivation:

Microgafted plantlets was subsequently maintained in culture in vitro on different types of media, but often poor or lacking phytohormones, and held for one week in low light, humidity and temperature conditions, and after that intensity of thid exogenous factors was increased to the optimum for that species. .

The micrografting success was determined trough check point welding. It was found that micrografting success ranged from 10-80% depending on genotype. 8 weeks after grafting the bud development its began and at that time reached a length of 5 mm to 12mm

The good contact between partners tissue is one of the essential factors for grafting success. If there was movement from initial position of microscion in some cases was formed a calogen tissue that filled the space between rootstock and scion, and graft union was formed during a longer time. However, some combinations have not been able to restore the connections between partners. Such microscions were brunificat and died. Most authors considered the callus formation as an indicator of the success of grafting because callus formation between graft and rootstock fill gaps and ensure circulation of fluid between partners before differentiation of vascular tissue (Hartmann et al. 1997). If movement of microscion is too large dehydration occurs, and in this case callus is not formed and microscion die (Ramanayake and Kovoor 1999) Success rate in our experiments is relatively high compared with that obtained from *Citrus* 30–50% (Navarro et al. 1975).

Since graft incompatibility can be detect after years of plants growing, for early detection of this process it can successfully used the callus co-culture technique. (Jonard et al. 1990; Errea et al. 2001. Callus cells are able to eliminate the partner's cells at early stage of development, and in this case we can talk about a incompatibility response. Cell recognition is following by cell differentiation (Considine 1983) and callus cells can initiate tissue continuity at the compatible partners and necrosis at the incompatible ones. (Pina and Errea 2005). Cell necrosis may occur in the case when parts of different origins

callus grown together for a period (Ermel et al. 1997), but can also appear in any stage of co-cultivation, especially on intense proliferation tissue. (Moore 1986).

In this context callus co-culture can be used as a biological model for studying the graft incompatibility phenomenon.

To initiate the callus cultures we have used different types of explants: seedling parts (hypocotil, epicotil), and petiole Sterilization of explants was performed with 70% alcohol for 30 sec. and sodium hypochlorite solution 5% for 5, 10, 15, 20 minutes. Sterilized explants were placed on MS culture medium with different hormone balances and the subcultivation on the fresh media was made in 14 days.

Fig 2

Acumularea de biomasa pe parcursul subcultiv rii calusului de origini diferite pe mediul de cultura B5+1mg/l 2,4D+1mg/lK

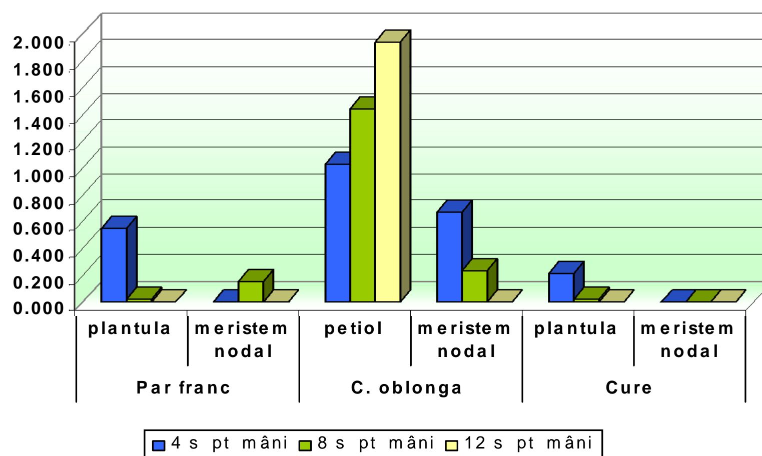


Fig.3

Acumularea de biomasa pe parcursul subcultiv rii calusului de origini diferite pe mediul de cultura MS+1 mg/l 2,4D+1mg/lK

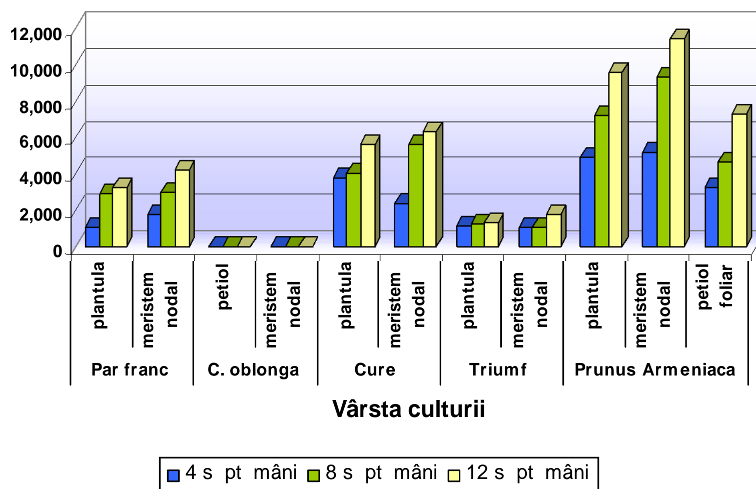
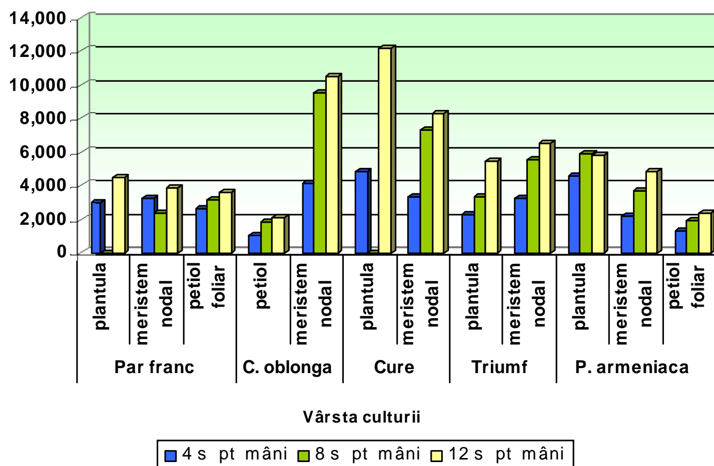


Fig.4

Acumularea de biomasa pe parcursul subcultiv rii calusului de origini diferite pe mediul de cultura G+1mg/l2,4D+1mg/lK



Profile of growth and development of callus cultures is not known if other than microorganisms. After sub culturing of the fresh media follows a lag phase during which the cells regain the ability of division, followed of an exponential phase, which involves rapid cell division and has a variable duration of time, witch depends on the type of nutrients in the culture medium.

. At the end of exponential phase cell division rate begins to decline, the growth curve via linear and the gradual decrease phases reached stationary phase. In general, this growth model can be pursued regardless of the methods of research and growth monitoring. There are several methods for measuring crop growth: determination of cell number, fresh or dried biomass, cell volume determination. . The most popular method remains, however, fresh or dried biomass determination. Daily accumulation rate (RAZ) of biomass of the studied genotypes recorded values which reflect the dependence of this parameter to interplay between nature of explant and culture medium composition. Biomass accumulation rate is the lowest first subcultivation, due to adaptation of cells, and then increased during subcultivation .

Biomass determinations were made at each subcu ltivation. The results represent the average of five samples per variant, and growth index was calculated from the formula: ΔG / GI, where GI - initial biomass. Consistently the best results were obtained on MS medium, which after the second subculturing callus growth index exceeded 150% at all variants and the maximum value of growth index (266%) occurred at callus of Cure variety aged 12 weeks, obtained from mechanically injured seedlings grown on MS medium.

Growth index varies very widely, dependi ng on the age and genotype of callus culture. The primary callus reveals a relatively high index of growth due to tissue hydration phenomenon onset installed on the debut of callusogenesis process.

.. Also on this level there is a visible difference betwe en the evolution of different explants, what difference is blurred because of the loss the link between explains and calusogen tissue and their adaptation to new life condition during subcultivation.

Recent research in this field indicates that the pheno menon of incompatibility may not be reduced to the morpho -physiological changes that occur to grafting point. Interaction between scion and rootstock drive changes of the metabolic processes witch taking place at this level. Involvement of enzymes (of cat alase, peroxidase, phosphatase) in the process of incompatibility of grafted trees was studied by many authors (Quesada and Macheix, 1984; Deloire and Hebant, 1982; Schmidt and Feucht,1985; Fernandez - Garcia et al., 2004), but this factor is not to be full y elucidated. Recently studies have been undertaken to establish biochemical markers to highlight the incompatibility phenomenon. Lachaund (1975) suggests that incompatibility could be avoided by choosing partners with similar protein composition. They mad e comparisons between the protein profiles of different species of Prunus in order to establish incompatibility before grafting (Huang et al., 1984; Schmid and Feucht, 1985). Content of total soluble proteins in different combinations with different level of compatibility grafts was studied by Moreno et al. (1994). They detected a lower amount of soluble proteins in incompatible combinations than in the compatible.

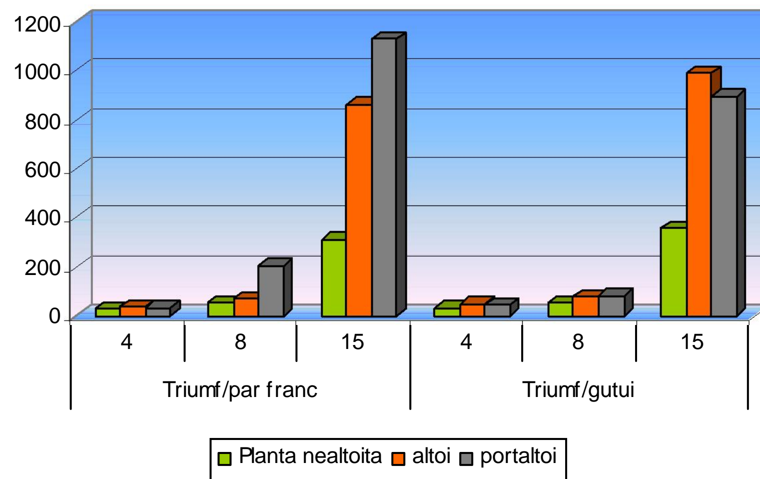
Although cell necrosis caused by hydrocyanc acid in contact surface of pear grafted on quince was demonstrated since 1968 by Gur and colab. the molecular basis of grafting process remained unclear until now. Interaction between scion and rootstock can be adjusted by growth regulators. Auxins play an important role in the successful merger of the graft partners because they are involved in the differentiation of vascular tissues (Moore, 1984; Aloni, 1987; Mattsson et al., 2007).

In this project we proposed plant biochemical characterization both in vitro micrografted plants and of callus obtained for co-culture technique. The following parameters were determined: catalase and peroxidase activity and total protein content, following that after obtaining sufficient material for analysis to quantify and other biochemical parameters.

Peroxidase activity was determined spectrophotometrically by estimating the increase of absorbance at 470 nm. Reaction mixture was composed of 50 mM potassium phosphate (pH 6,8), 10 mM hydrogen peroxide , 9 mM guaiacol and enzymatic extract in a total volume to 3mL, accord ing the method descrybe by Olmos et al. (1997). Determination of catalase was performed by the method described by Aebi (1984). Reaction mixture was composed of 50 mM potassium phosphate (pH 7·8), 10 mM H_2O_2 and enzymatic extract . Enzyme activity was determined by estimating the absorbance at 240 nm. Extinction coefficient was 36 mm -1 cm-1. Total protein content was determined by Bradford method. Catalase and peroxidase activity was determined in the two parts of micrografted plant: scion and rootstock highlight the involvement of these parts in building cohesion at the grafting point. Peroxidase activity increased during plant development. In all cases examined it was higher in grafted plants than in those not grafted. . At 8 and 15 days semnficative differences were observed between enzymatic activity and grafted rootstock, being significantly higher in the rootstock. In terms of catalase activity that was slightly different compared to those not grafted grafted plants 4 days after grafting. In contrast to 8 days after grafting catalase activity i n grafted plants was three times higher than those not grafted. After the 15th day is the grafting catalazel differences in activity and those not grafted grafted planets fade. Also, no significant differences observed on catalase activity in the graft and rootstock.

Fig. 6

Variation of peroxidase activity during plant development of in vitro micrografted plants

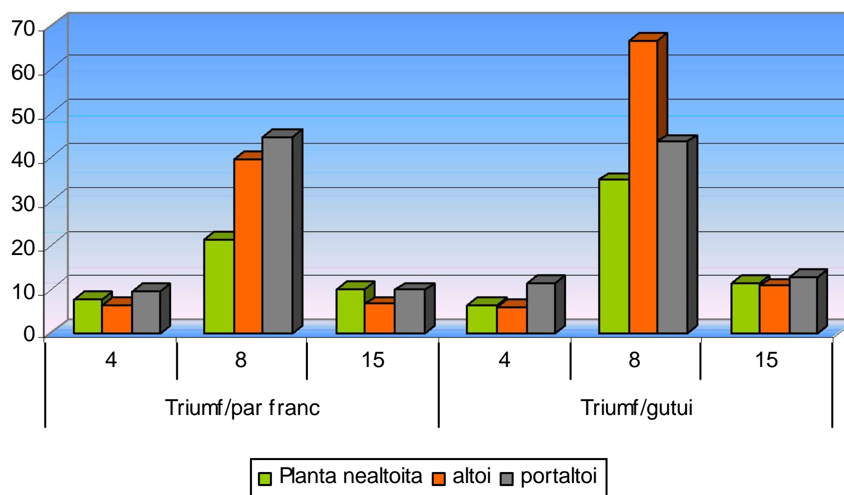


The early stage of development of cohesion between graft and rootstock, which takes place four days from mycrograf ting is characterized by destruction of the cells from the grafting surface and theyr reactions to injury (Moore, 1984; Tiedemann, 1989), followed by calogen tissue generationat the grafting surfaces of both partners. Callus is generated based on intact parenchimatic cells, but also on the medullary rays and phloem parenchyma (Jeffree and Yeoman, 1983). On the incompatible combinations can be seen forming a lack in the pith level of rootstock and dehydration of injured tissue. . Necrosis of medullary rays subsequently observed both in scion and the rootstock. this phenomenon was found both in incompatible and compatible micrografted combinations, so it can be concluded that the early development of callus formation is a general response to injured tissue. Differentiation of new leading vessels from parenchymatic cells occurs between the day 5th and 8th and lasts approximately 15 days for their complet restauration. (Stoddard and McCully 1979)

Several authors have demonstrated the peroxydase involvment in the lignification processes (Whetten *et al*., 1998; Quiroga *et al*., 2000). The results obtained by us show an increase of peroxydase activity during the development of grafted plants. Also, graft ed plants had a higher enzyme activity than those not grafted. These results demonstrate the development of lignification processes at the grafting point and their enhance towards the end of the second week after micrografting. It was found that lignificat ion occurs continuously throughout the formation of cohesion, since the 4th day, on the small areas. At this stage it is apparent and increased peroxidase activity. The results show that active biosynthesis of lignin begins the early days of grafting. At 8 th day the most of the lignin biosynthesis activity is noted in the graft, which is reflected in an increase peroxidase activity.

Fig. 7

Variation of catalase activity during plant development of in vitro micrografted plants



The highest catalase activity was recorded in the 8th day.

This activity is generated by an increased quantity of hydrogen peroxide accumulated in the tissues, in the stress conditions that accompanies grafting process. (Bestwick *et al*., 1997; Orozco-Cardenas and Ryan, 1999; Pellinen *et al*., 2002).

Hydrogen peroxide can be a byproduct of the lignifications process, but also a reaction to stress conditions. The high catalase activity in our experiments may be associated with intense proliferation of callus cells as a result of injury reaction .. Finally, it can be concluded that catalase and peroxidase activity are closely correlated spatially and temporarily and and is sensitive enough to by considered an biomarckers f or cell differentiation processes at early stage of grafting

Peroxidase activity and total protein content was determined in the callus being at its third subculturing on MS medium with different phytohormonal balance. The highest enzymatic activity in all studied species was detected in callus cultivate don media with low auxine content. (Varianta 1 si 2). The highest protein content was obtained on media with high auxine content but without citokinines. (V3). These results reflect the dependence of these indicators to dediferentietion and rediferentiation processes occurring in the callus and influenced by phytohormonal compozition.

It is generally known the involvement of auxines in organogenesis processes . On the other hand, peroxidase is also inv olved in degradation of 2,4 D with formation of two compounds witch migrating to apex of the plants and leading to organ development. (LESNEY, 1990). So, auxine excess lead to inhibition of peroxidases activity. It was found that on the media supplement ed with BAP enzymatic activity was lower than on media supplemented with kinetine. Considering that the BAP has generated a greater accumulation of callus it might conclud e that peroxidase activity is inversely proportional to the ability of callus proliferation.

Fig. 8

Variation of the peroxidase activity of callus produced on MS medium with different growth regulator ratio

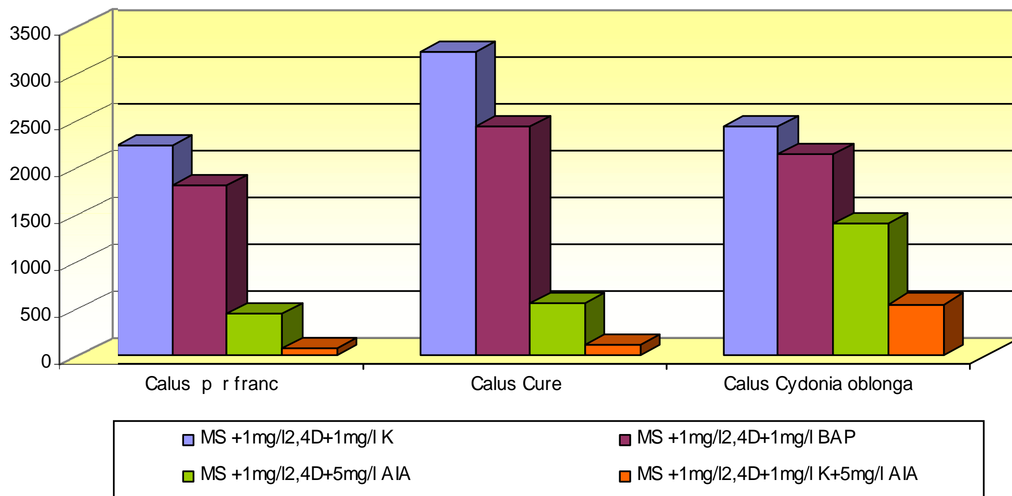


Fig. 9

Variation of the catalase activity of callus produ ced on MS medium with different growth regulator ratio