USE OF UNCONVENTIONAL METHODS TO OBTAIN SOMACLONAL VARIATIONS, WITH THE PURPOSE OF CREATING NEW POTATO VARIETIES THAT ARE RESISTANT TO DISEASES AND PESTS

Andreea TICAN, Nicoleta CHIRU, Diana IVANOVICI
Department of vegetal tissue culture
National Institute of Research and Development for Potato and Sugar Beet Brasov, Romania

*** Correspondence:** Andreea Tican, Institutul Naţional de Cercetare Dezvoltare pentru Cartof şi Sfeclă de Zahăr Braşov, str. Fundăturii nr. 2, tel: 0268/476608, fax:0268/476795, e-mail: tican_andreea@yahoo.com
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ABSTRACT. The most common factors which affect somaclonal variations are genotype explant source, in vitro period and cultivation conditions in which the cultureis established. In this research, calli were induced using leaf disk and leafstalks explants of potato cultivars resistant at potato blight and viroses. Calli were induced with different growing regulators (with different concentrations). Having as objective obtaining of lines with resistance at diseases and pest are necessary: callus induction, callus growing, callus multiplication by subculture, controlling organogenesis processes. Plantlets regeneration from callus is influenced by genotype ad medium variant.

Keywords: somaclonal variations, callus, tissue culture, induction, medium, genotype, type of explant

INTRODUCTION

Potato (Solanum tuberosum L.) is one of the most cultivated vegetable in Romania and the world's fourth most important crop (Solomon-Blackbourn & Barker, 2001). Potato is a tetraploide species and using of botanical seeds in commercial cultivation is impossible, owing to low germinability and large variability in segregant generations. Synthetic seed is an alternative in solving of this problem. Original definition of synthetic seed is "a single encapsulated somatic embryo" (Murashige-1978). Synthetic seed is a clonal product, which can be handle and used as a real seed in transport, storage, "ex vitro" and also for "in vitro" planting. The most accepted definition of synthetic seed is: "artificially encapsulated somatic embryos, sprouts and other tissues that can be used *in vitro* or *ex vitro* plantings.

Isolated cell tissue cultures (even from one cell) may present variation after repeated subculturing. Distinct lines may be selected with their particular morphology. This variation (somaclonal variation) may be send to regenerated plants from culture tissue. This is an additional source of a new variation for breeders. Somatic embriogenesis produce somatic embryos and can be used for the production of synthetic seed. Somaclonal variation may be associated with somatic embryogenesis (Patricia N. Bordallo *et al.* 2004).

Somaclonal variation is a common phenomenon on cell plants cultures, including all variation types among plants and cell and derives from all kinds of tissue cultures [(Skirvin *et al.* 1993) quoted by (Patricia N. Bordallo *et al.* 2004)].

Somaclonal variation is also called induced variation of tissue or cultures. Because the production goal of synthetic seeds is to obtain clonal identity, to control somaclonal variation became a challenge ((Amirato, 1991) quoted by (Patricia N. Bordallo *et al.* 2004)).

Many causes have been identified or proposed for each type of variation; these however, may very from species to species and determining the genetic nature of the observed variation is difficult (Maraschin *et al.* 2002) (Patricia N. Bordallo *et al.* 2004)).

These variation causes include: changes in the structure and/or chromosome number, noticeable point mutations, changes in the expression of a gene as a result of structural changes in the chromosome or activation of transposable elements, chromatin loss, DNA amplification, somatic crossing over ((Rao et al., 1992; Kaeppler, et al., 2000). There are four critical variables for somaclonal variation: genotype, explant origin, cultivation period and the cultural condition in which the culture is made (Patricia N. Bordallo 2004 *et al.* 2004).

Somaclonal variation is very important in breeding, because it can took place changes to vegetal material coming from value varieties, and then by selection, are holding clones which kept important characters, and besides that, followed characters for being improved (Bianu T. *et colab.* 1989).

Plant genotype may have important effects on somaclone regeneration and frequency. These effects are very evident on potatoes: differences are observed in the number of regenerated plants of distinct cultivars grown under identical conditions (Gunn & Shepard, 1981).

Explant source is considered the most frequent critical variable for somaclonal variation. Procedure selection of regeneration depends by different type of explants. Somaclonal variation is influence by genotype, tissue source, and procedure and culture time realization. In present, for obtain somaclonal variation is used a certain number of techniques and tissue cultures.

Among tissue culture techniques, callusogenesis becomes an objective aimed at initiating callus cultures or inducing somaclonal variability. At this level the genome altering may involve numerical aberrations or chromosomes rearrangements as well as genes mutations (Larkin *et al.* 1981). The genetic variability is generated through cell and tissue culture and the regenerated plants and their descendants inherit more, this variability (Chiru N. *et al.* 1994).

The callus, after is induced, must have the capacity to generate a big number of plantules (Chiru N, 1998).

Somaclonal variability is also important for obtain parental forms with special resistance features which may be introduced in a breeding scheme through classical or somatic hybridization (Chiru N *et al.* 1994).

Inducing *in vitro* culture cycle of a callus phase increases significant somaclones frequency. Relating to new solutions it can be specified that medium with a composition on which is predominant auxine (in special NAA), citochinine (zeatine) with different concentration may be a cause of abnormal appearance to potato plants.At potato, explants origin represents other source of somaclonal variation. Existing of "medium microconditions" (osmotic pressure, light, different substance-natural mutagen) may induces hereditary changes in cells.

Starting with necessity of having varieties resistant to diseases and pests, our researches will be directed in trying of obtain resistant lines using as method plant neogenesis, and having as staring point callus.

This suppose:

- callus induction,
- callus growing;
- callus multiplication by subculture
-controlling organogenesis processes (Chiru N, 1998).

MATERIAL AND METHODS

In National Institute of Research and Development for Potato and Sugar Beet (INCDCDZ) Brasov, our research have been directed to plants' regeneration from *in vitro* tissue culture and analysis of factors which compete to their realization:

- genotype
- explant source
- phytohormonale composition
- cultures conditions
- medium
- sterilization of biological material

Sterilization of biological material, harvested from the green house, was
accomplish in this way:

- washing on water jet: 5-7 minutes;
- sterilization in mercuric chloride (1%) for 10 minutes;
- washing of biological material in distilled water, for three times, in three waters;
- drying of vegetal material on sterile filter paper (Chiru N., 1998)

Explants was represented by a leaf disk (1 cm^2) and leafstalks segments, (1,5-2 cm length), which was drawn from 4 potato genotypes (table 1). After accomplishing the disinfecting, biological material was transferred in Erlenmeyer flasks, in position with normal polarity, containing a base medium: Murashige-Skoog (1962) with vitamins, 30 g/l sugar, 8 g/l agar and different growth regulators, of different concentrations (table 2).

Hormonal composition of culture medium is reproduce in table 2, in function of different growing regulators and their concentration. pH was 5,8, and medium was sterilized by autoclaving, at 121^0C, for 20 minutes, at 1,25 atmospheres. Explants drawing were accomplished with laminar flow.

It was inoculated 20 explants/genotype for each medium variant, and after inoculation, cultures were transferred in growing room, in dark conditions for first 2 weeks, with temperature of 23^0C. After this period of time, cultures were maintain at 24^0C, with a photoperiod of 16 hours of light and 8 hours of darkness.

It was used varieties resistant to virosis (Christian and Roclas) and lines resistant to potato blight R0 99 SASA and R1 99 SASA. Our objective was to obtain somaclonal variation with resistance to potato blight and virosis.

Table 1

USED GENOTYPE

Genotype	Type of explant
R0 99 SASA (Belgia)	Leaf disk, Leafstalks segments
R1 99 SASA (Belgia)	Leaf disk, Leafstalks segments
CHRISTIAN Cl.1 (Lăzarea)	Leaf disk, Leafstalks segments
ROCLAS Cl.2 (Lăzarea)	Leaf disk, Leafstalks segments

Table 2

COMPOSITION OF CULTURE MEDIUM FROM POINT OF VIEW HORMIONAL FOR CALUS INDUCTION FROM DIFFERENT POTATO EXPLANTS

Medium variant	Used explant type	Growth regulators (mg/l)						
		BA	NAA	AG_3	Kin	Zeatine	IAA	2,4-D
C_1	Disk of foliar limb	0,5	2,0	0,1	-	-	-	-
C_2		-	1,0	-	0,5	-	-	-
C_3		-	-	-	-	0,2	2,0	-
C_4		0,5	-	-	-	-	0,5	3,0
C_5		6,0	2,0	-	-	-	-	2,0
C_6		4,0	2,2	-	-	-	-	4,0
C_7		6,0	2,2	-	-	-	-	4,0
C_8		4,0	2,0	-	-	-	-	2
C_9	Leafstalks segments	4,0	2,2	-	-	-	-	4,0
C_0		-	-	-	-	-	-	-

RESULTS AND DISCUSIONS

From existing material in greenhouse, in plant vegetation phase, it was manual harvested biological material from aerial part of plants (from leaves and steams). This was tested, as we written in chapter materials and methods. For the first phase, our research was directed to induce callus from inoculum and in the second phase (fig. 1 and 2), it was tried a regeneration of plants from obtained callus. We had in view genotype influence on callus induction and influence of different medium variants used.

It was observed positive answer of both factors for callus proliferation.

Callusogenesis was developed after 8 weeks from inoculation, and regeneration of plantlets from callus, took place at 12-14 weeks (since explants inoculation).

Fig. 1 Callus induction from leafstalks segments

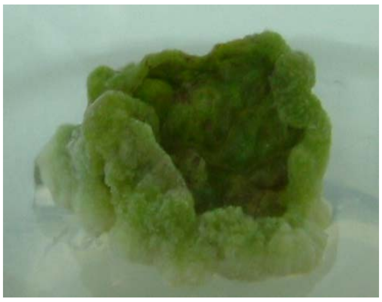


Fig. 2 Callus induction from leaf disk

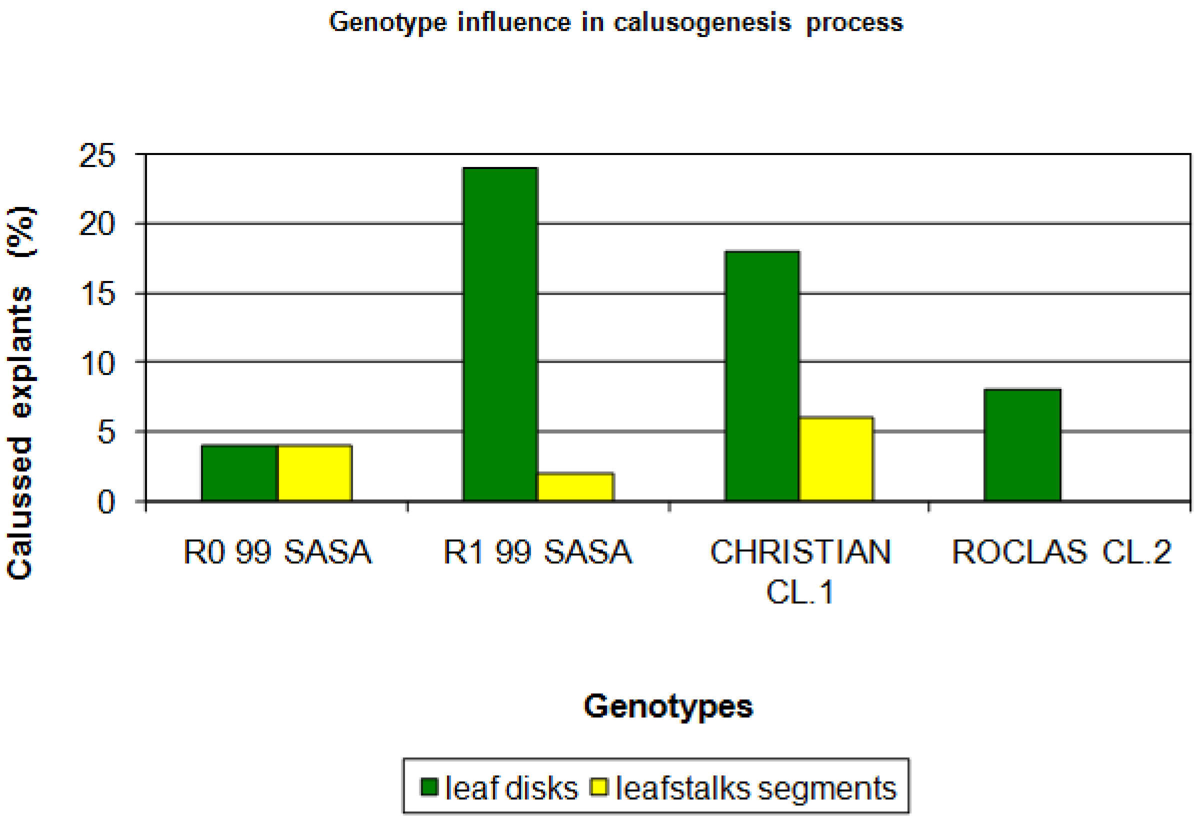
Fig. 3 Plantlets regeneration from callus

Table 3

GENOTYPE INFLUENCE AND INOCULUM TYPE ON CALLUS INDUCTION, AFTER 8 WEEKS FROM INOCULATION

Genotype	% of callus induction	
	Leaf disk	Leafstalks segments
R0 99 SASA (Belgia)	4	4
R1 99 SASA (Belgia)	24	2
CHRISTIAN Cl.1 (Lăzarea)	18	6
ROCLAS Cl.2 (Lăzarea)	8	0

Fig. 4



Genotype influence on callusogenesis process shows R1 99 SASA line (Belgium) (fig.4), as important genetic source. The productivity of this line is big in callus proliferation (24%) from explants of leaf disk and is lower (2%) for explants from leafstalks. Line R1 99 SASA has resistance to potato blight and we can note that proliferate in a big percent (24%). Also line R1 99 SASA has resistance to potato blight, but it proliferate in a smaller percent.

Christian variety was distinguished in callus production for leaf disk (18%) and for leafstalks segments (6%). We can observe importance of this early variety for establishing genetic source in calusogenesis process. We want to mention that Cristian is resistant to viroses, and so it will be posile to create somaclonal variation with resistance to viroses.

Even if the line R0 99 SASA (Begia) presented in our research a small rate for callus proliferation (4%), this can be an important material in calusogenesis process, because the callus came from leaf diks explants and leafstalks explants.

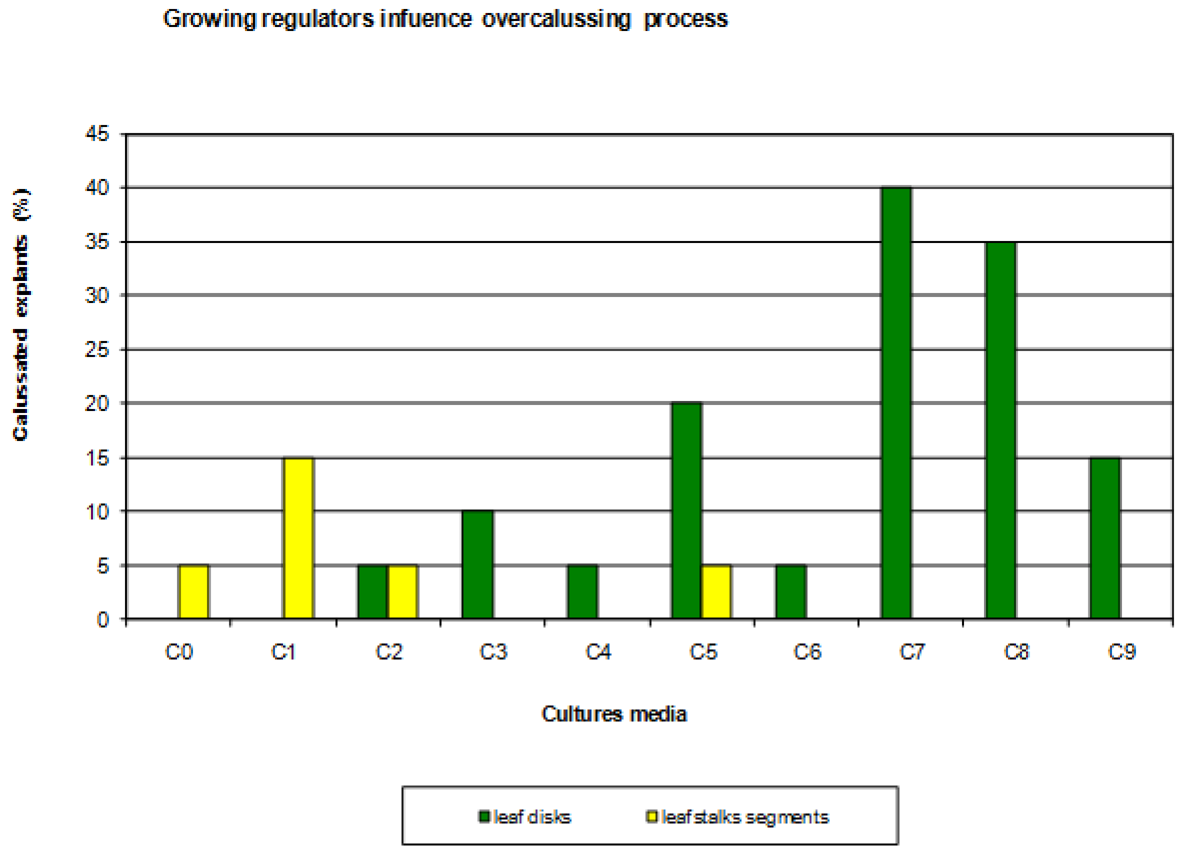
Early variety Roclas, it is the only variety, which in calusogenesis process proliferated callus from leaf disc, with an intensity of 8%.Our goal is to obtain new varieties with resistance to diseases, pests.

Table 4

GROWING REGULATORS INFLUENCE OVER CALLUSING PROCESS OF EXPLANTS (%)

Medium variants	Leaf disc	Leafstalks segments
C0	0	5
C1	0	15
C2	5	5
C3	10	0
C4	5	0
C5	20	5
C6	5	0
C7	40	0
C8	35	0
C9	15	0

Fig. 5



We can observe (fig. 5) about the influence of growing regulators in callusing process, the presence of callusing process on 8 cultures medium variants (for leaf disk) and for 4 culture medium variants (for leafstalks segments). The biggest callusing percent (40%) was observed on medium culture variant C7, for explants with leaf disc (containing 6 mg/l BA, 2,2 mg/, NAA, 4 mg/l 2,4 –D). This was followed by culture medium variant C8 (35%), containing 4 mg/l BA, 2,20mg/, NAA şi 2 mg/l 2,4 –D. It could remark rising of callusing percent once with rising of BA content at 6 mg/l and NAA rising until 2,2 mg/l. After this, we can see a reduction of callus percent at reduction of content in BA and NAA. The values achieved was comprise between 5% and 40%, for leaf disks explants.

About the maximum intensity of callusing proces on explants from leafstalks segments, we note that this took place on culture medium variant C1 (15%-containing 0,5 mg /l BA , 2,0 mg/l NAA and 0,1 mg/l AG_3), followed by medium variant C0 (testefier medium), C2 (5%-containing 1 mg/l NAA + 0,5 mg/l kinetine), C5 (5%, with a content of 6 mg/l BA, 2,0 mg /l NAA , 2,0 mg/l 2,4 – D).

Analyzing the influece of (fig. 6a) tested genotype over callus production, dependenting on used medium variant, we can note that line R0 99 SASA, produce callus from explants proceed from leaf disk (in ratio of 40%) for medium variant C7 (containing 6 mg/l BA, 2,2 mg/, NAA , 4 mg/l 2,4 –D). Callusing reaction from leafstalks segments explants was observed only for testifier culture medium. This contains Murashige-Skoog medium (with vitamins), 30g/l sugar, 7g/l agar, 0,5 ml/l NAA for medium variant C1 contain 0,5 mg /l BA+2,0mg/l NAA+0,1 mg/l AG_3 (in the same percent 20%).

Line R1 99 SASA, (fig. 6b) had a positive reaction on callusogenesis for 5 variants. It grow with an intensity from 20% (for medium variant C2) to 40% (C3), followed by absence of proliferation at medium variants C4, C5, C6. C7 had the strongest growing of the callusing process (80%), containing 6 mg/l BA, 2,2 mg/, NAA, 4 mg/l 2,4 –D. After that, we note diminution of callusing percent owing to medium variant C7, medium variants C8 (60%) and C9 (40%). This were constitute from 4 mg/l BA, 2,20mg/, NAA and 2,0 mg/l 2,4 –D (C8) and 4 mg/l BA, 2,20mg/l NAA, 4 mg/l 2,4 –D (C9).

Fig. 6A

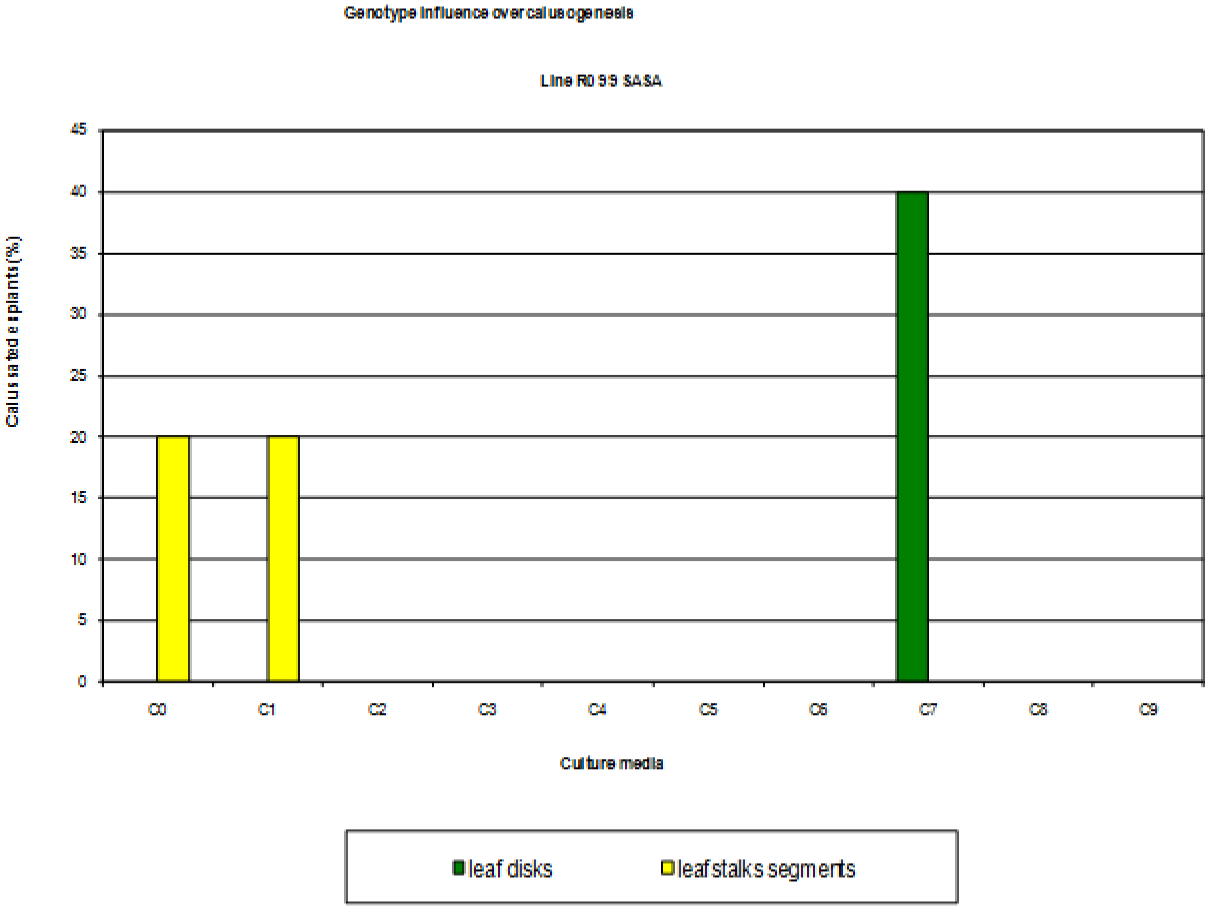

Genotype influence over calusogenesis
Line R0 99 SASA
Calus sated explants (%)
45
40
35
30
25
20
15
10
5
0
C0
C1
C2
C3
C4
C5
C6
C7
C8
C9
Culture media
leaf disks
leaf stalks segments

Fig. 6B

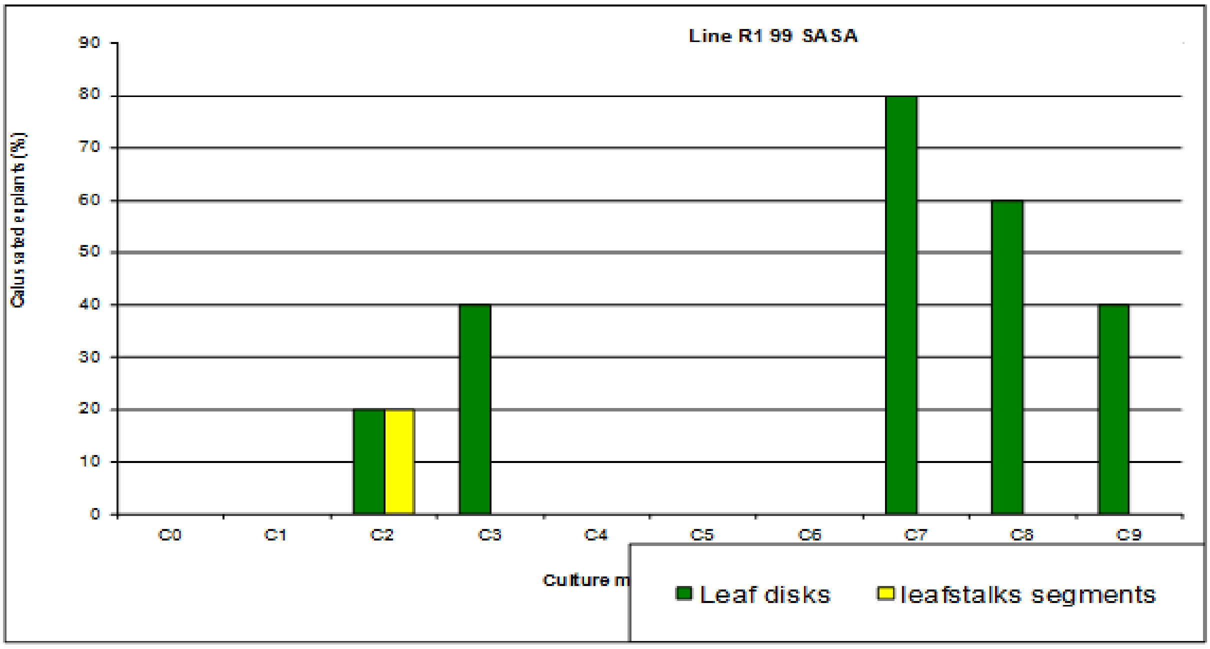
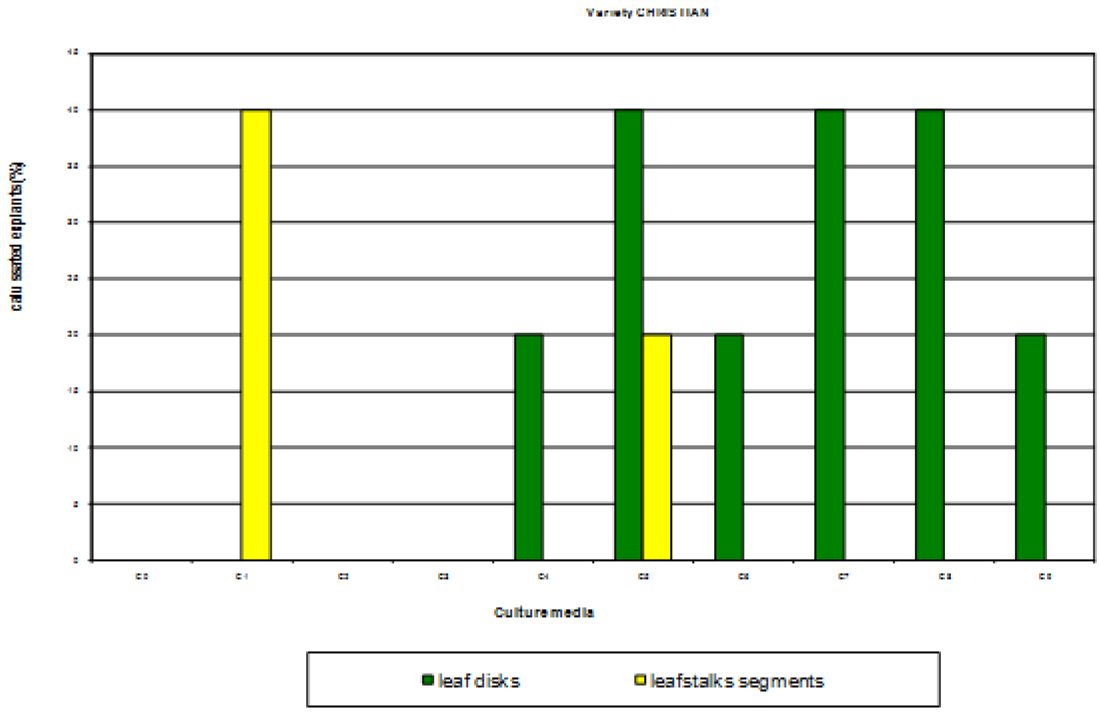
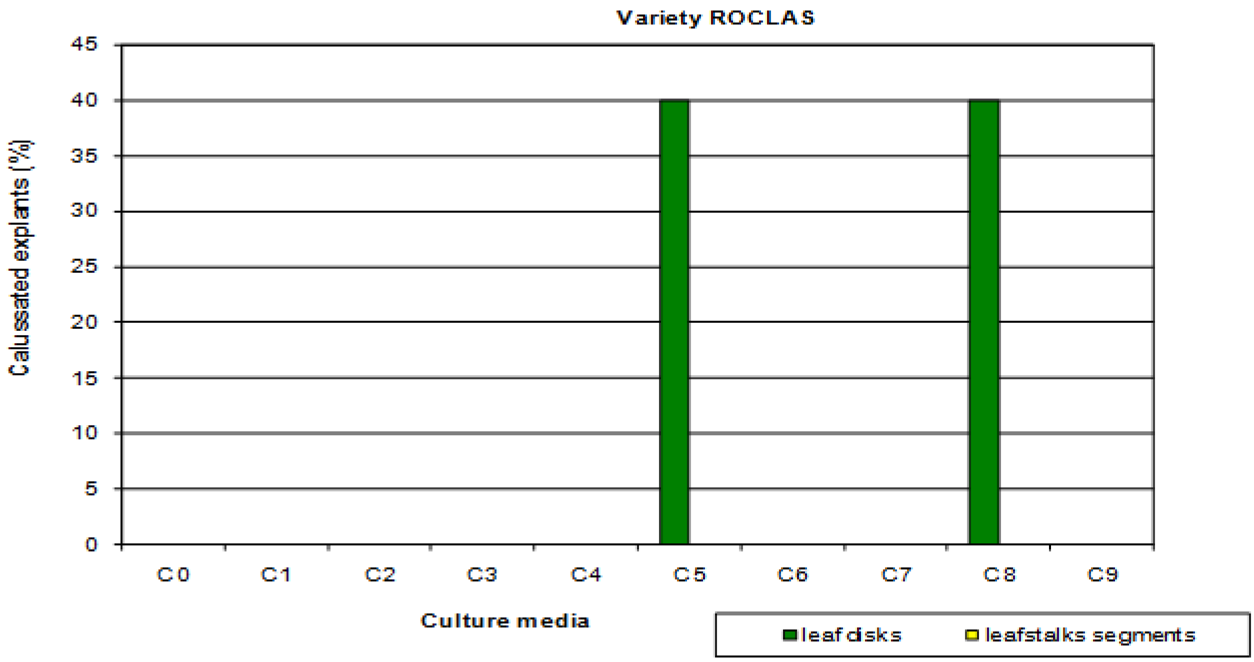

Line R1 99 SASA
Calus sated explants (%)
90
80
70
60
50
40
30
20
10
0
C0
C1
C2
C3
C4
C5
C6
C7
C8
C9
Culture m
Leaf disks
leafstalks segments

Fig. 6C



Variety Christian (fig. 6c) proves callusing capacity at both types of explants, but predominant at explants leaf disk for 6 culture medium variants and leafstalks segments for 2 medium variants. Maximum process intensity (40%), was note at medium variant: C1 (BA 0,5 mg, NAA 2,0 mg, AG_3 0,1 mg-leafstalks segments), C5 containing BA 6,0 mg/l, NAA 2,0 mg/l , 2,4 – D 2,0 mg/l, C7 containing BA 6,0mg/l, NAA 2,2mg/l , 2,4 – D 4,0 mg/l, C8 containing BA 4,0mg/l, NAA 2,0mg/l , 2,4 – D 2,0 mg/l-leaf disk. It is diminish at half the process intensity for medium variants C4 (BA 0,5 mg/l, IAA 0,5mg/l, 2,4 –D 3,0 mg/l), C6 (containing BA 4,0mg/l, NAA 2,2mg /l, 2,4 – D 4mg/l) and C9 (BA 4,0mg/l, ANA 2,2mg/l , 2,4 – D 4,0mg/l) for leafstalks segments. We can note culture medium variant C5 (BA 6,0mg/l, ANA 2,0 mg/l , 2,4 – D 2,0 mg/l). This determineted callus proliferation for leaf disk, also for leafstalks segments.

Fig. 6D



From complex analyse of growing regulators influence over explants of leaf disk and leafstalks segments, it can be establish:

Fig. 7

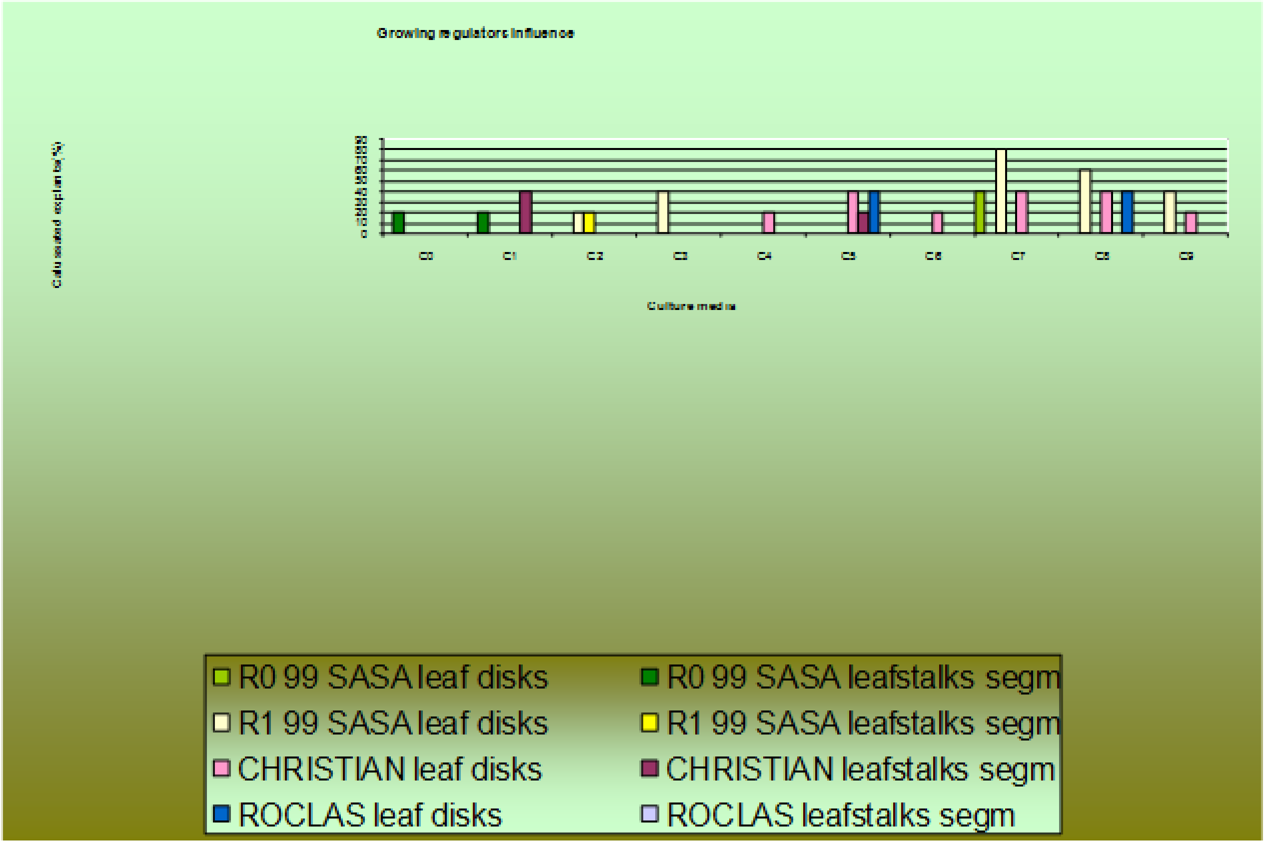
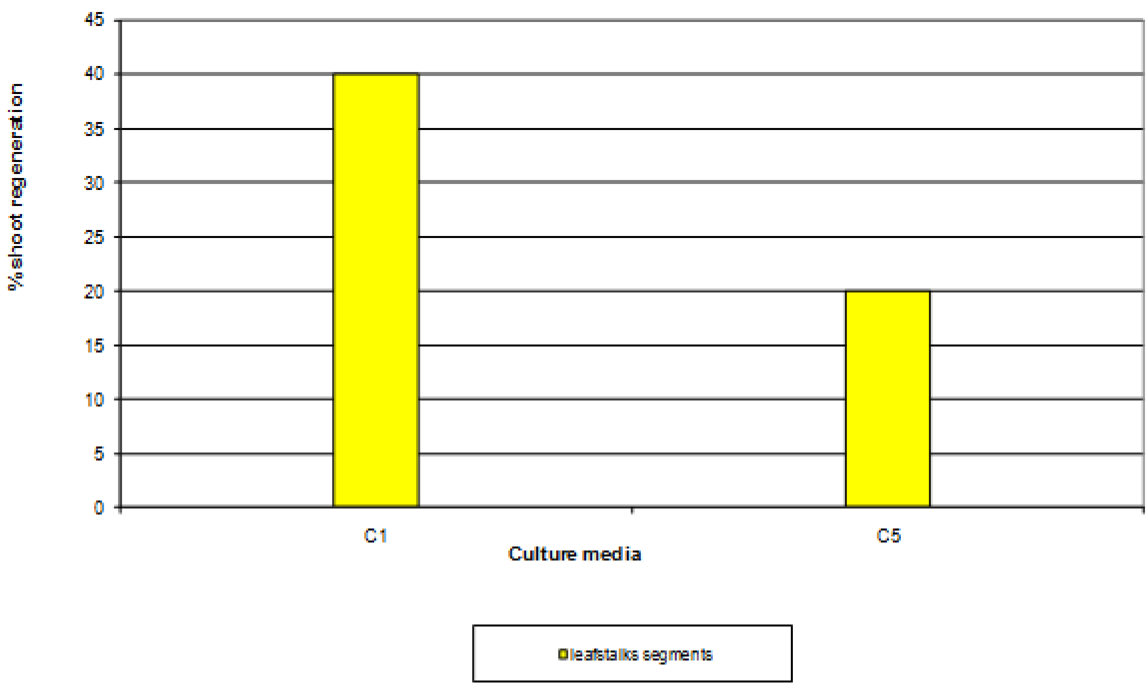


Fig. 8

Influence of culture medium over regeneration of shoot on callus coming from leaf disks for CHRISTIAN variety



The early variety Roclas (fig , proved the same calusogenesis capacity only for 2 medium variants (for C5 and C8, which contain: C5: BA 6,0 mg/l, NAA 2,0mg/l , 2,4 – D 2,0mg/l, C8: BA 4,0mg/l, NAA 2,0mg/l , 2,4 – D 2,0 mg/l) with 40% intensity for explants which come from leaf disc.

From figure 7 we may conclude:

1. All culture media induced callus, to all genotypes, with an intensity between 20-80%.
2. On 7 medium variants used, callusing process was at 20%, on 6 medium variants, callusing process was 40%, for one medium variant, the intensity was 60%, and for other one 80%.
3. On 2 medium variants, callusing process was find for leaf disk and leafstalks segments for 2 genotipes.
4. On 4 medium variants callus proliferation took place from leafstalks segments
5. On 8 medium variants, callus proliferation took place from leaf disk
6. On 3 medium variants, callusing process was present at 2 genotypes
7. On 2 medium variants, callusing proces was present at 3 tested genotypes
8. Line R0 99 SASA proliferated callus on 3 medium variants; variety Christian (resistent at viroses) proliferated callus on 7 medium variants, line R1 99 SASA (resistent al potato blight) proliferated callus on 5 medium variants; variety Roclas proliferated callus on 2 medium variants
9. Line R1 99 SASA had the biggest callusing percent on 2 medium variants (80% on C7 medium variant and 60% on C8 culture medium variant.
10. Genotypes R1 99 SASA and Christian induced callus on 2 medium variants for leaf disk and also for leafstalks segments.

Analyzing the posibility of shoots regenerations from callus (fig.8), we can note that early variety Christian generated shoots, only from callus coming from leafstalks segments on 2 from 10 medium variants used: C1 and C5 cotaining 0,5 mg/l BA, 2,0 mg/l NAA, 0,1 mg/l AG3 (C1) and 6,0 mg/l BA, 2,0 mg/l ANA, 2,0 mg /l 2,4- D(C5), with an breeding intensity reduced at half (20%).

At line R0 99 SASA (Belgia) (fig.9), it can be observe callus production on testifier medium variant and on medium variant C1 (0,5 mg/l BA, 2,0 mg/l NAA, 0,1 mg /l AG3). Proliferation intensity is the same forboth kind of medium (20%), but only from inoculum coming from leafstalks segments.

Fig. 9

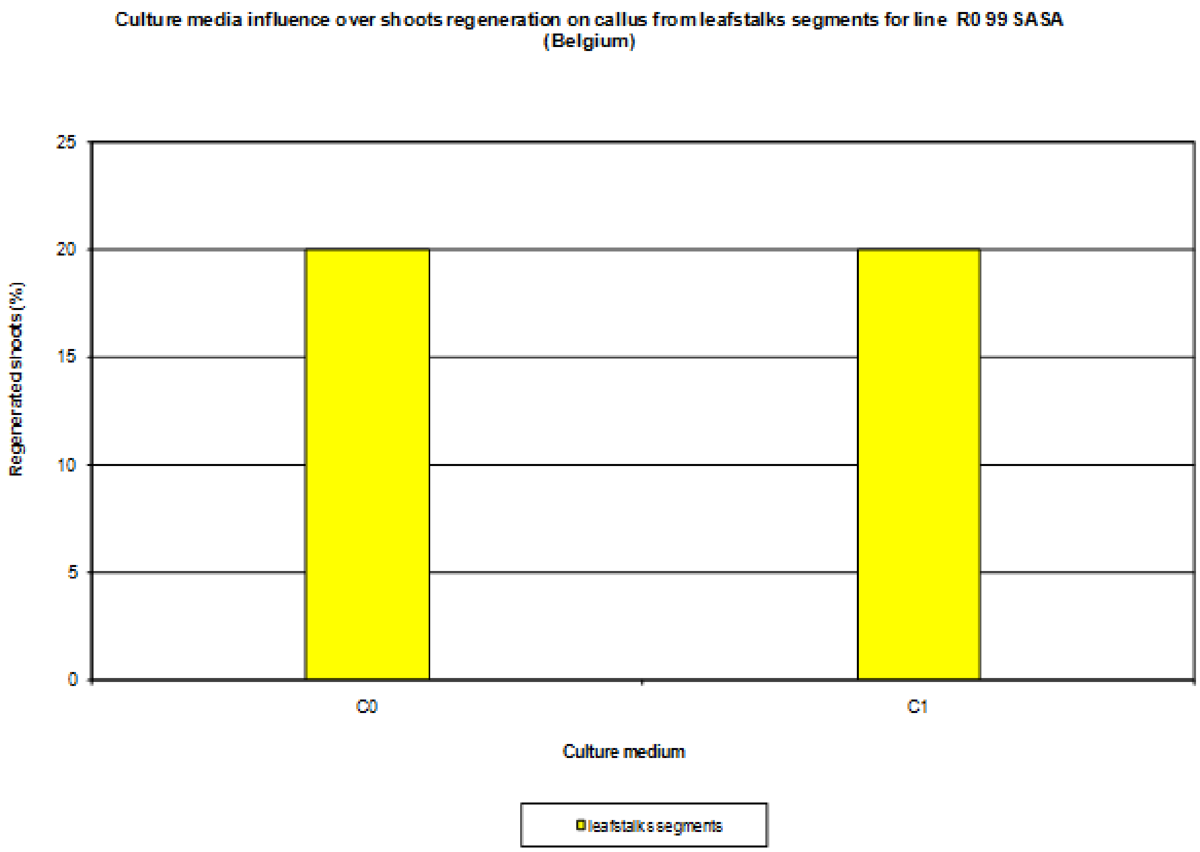


Fig. 10

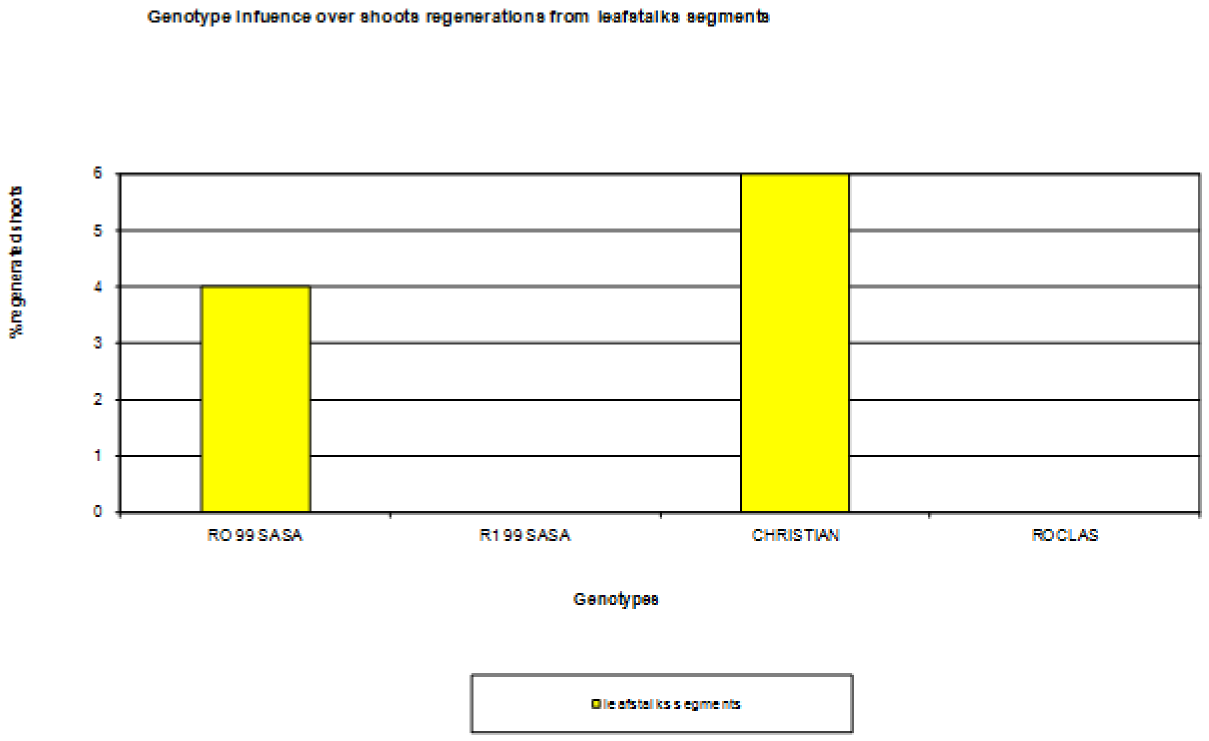
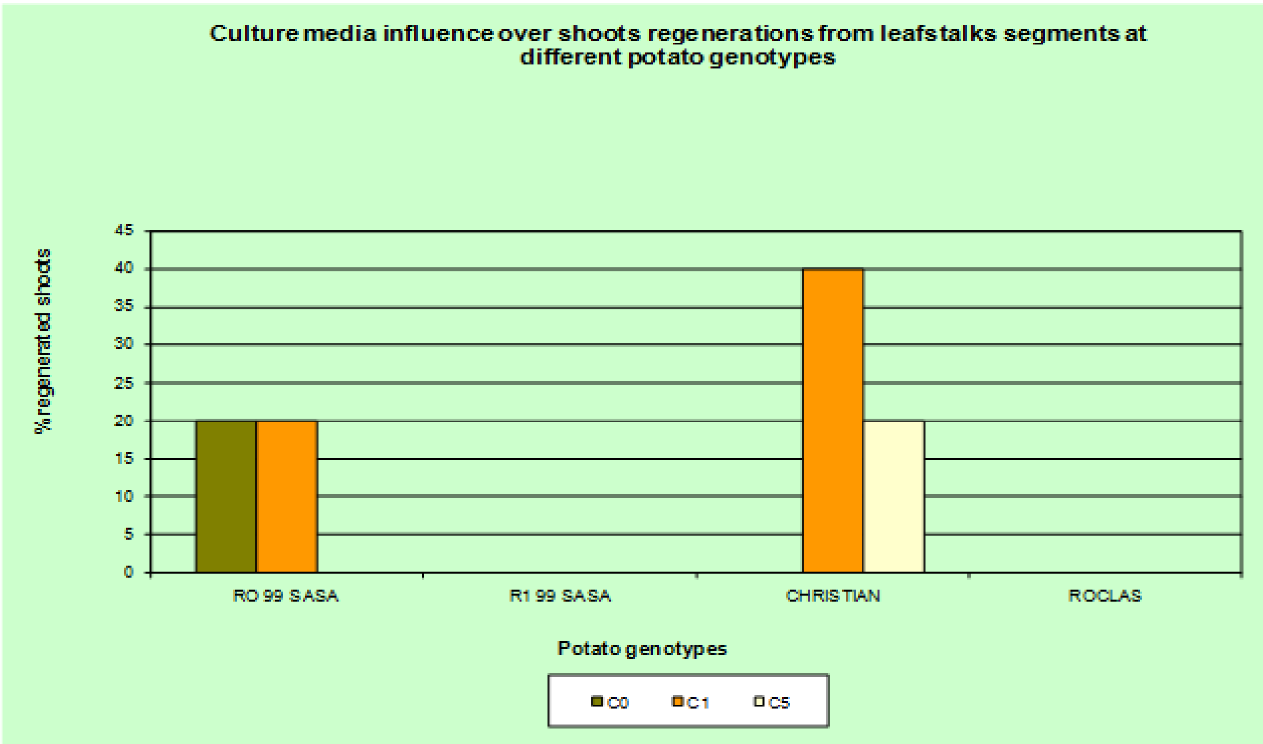


Fig. 11



Analyzing shoots producing capacity (fig. 10), it can be noted that R0 99 SASA and Christian presented callus regeneration from leafstalks segments, in a 4% percent and 6%.

Even if from medium variants it were produced shoots from leafstalks explants with a small rate, this proves that shoots regeneration it's possible on the future, supposing improving methods.

Christian (resistant al viroses) (fig.11) and R0 99 SASA (resistant at potato blight) regenerated plantlets on three medium variants: C0, C1 and C5.

Christian variety presented the best behavior for buds' regeneration, in a percent of 40%, from leafstalks segments produced callus. Regenerated plantlets developed in test tubes with MS culture medium, enriched with vitamins and 0,5 mg/l NAA, 20 g/l

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sugar, 8 g/l agar, with a 5,8 pH for medium variants (BA 0,5 mg/l, NAA 2,0 mg/l, AG_3 0,1 mg/l –C1; BA 6,0 mg/l, ANA 2,0 mg/l , 2,4 – D 2,0 mg/l –C5).

Line R0 99 SASA (resistant at potato blight) generated buds , even if in a small percentage than Christian variety (20%) on testefier medium variants C_0 (wit MS medium enriched with vitamins, 0,5 mg/l NAA, 20 g/l sugar, 8 g/l agar, with a pH 5,8) and C1 containing BA 0,5 mg/l, NAA 2,0 mg/l, AG_3 0,1 mg/l.

CONCLUSIONS

For potato callus induction, is necessary the presence in culture medium of growing regulators – auxine and citochinine in a equilibrate proportion from concentration point of view.

Callus induction take place at inoculum coming from leafstalk segments or leaf disc, with a biggest percent proliferation for inoculum coming from leaf disk.

Plantlets regeneration from callus is influenced by genotype ad medium variant.

Is possible to create new varieties with resistance at potato blight and viroses.

Is necessary to continue the research about callus induction and plants' regeneration, by testing much more varieties and using other kind of different medium by their composition and their concentration.

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