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Influence of Genotype, Explant Type and Hormonal Composition of Culture Medium on in Vitro Callogenesis of Citrus Species

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***Abstract* – In order to establish optimum conditions for callogenesis of citrus species, the effects of some factors, namely the genotype, explant type and hormonal composition of culture medium on callus induction were studied using eight cultivars: Amblycarpa (*C. amblycarpa (Hassk.) Ochse*), Cleopatra mandarin *(C. reticulata blanco)*, Carrizo citrange (*Citrus sinensis (L.) Osbeck X Poncirus trifoliata (L.) Raf.*), Hamlin orange *(Citrus sinensis (L.) Osb.)*, Rangpur lime *(Citrus x limonia Osbeck)*, trifoliate orange *(Poncirus. trifoliata)*, Troyer citrange *(C. sinensis X P. trifoliata*) and Volkamer lemon *(Citrus Volkameriana)*. The present work focused particularly on the ability to induce friable calluses from three types of flower explants: styles, stigmas and ovules. These were sampled from mature trees before anthesis and cultivated on MS or MT medium (Murashige and Skoog) supplemented with malt extract, saccharose, agar and phytohormones including 2,4-dichlorophenoxyacetic acid (2-4D), 6-benzylaminopurine (BAP) and Kinetin (Kn). Callogenesis, which started after 15 days of dark incubation, was shown to be the most successful when using styles of Troyer and Carrizo citranges as explants and MS medium containing the hormone combination: 1 mg.l^{-1} 2, 4-D+0.5 mg.l^{-1} BAP. However, taken together, our results suggest that ovules are more suitable explants for callus induction than styles and stigmas since these resulted generally in higher callogenesis rates in all the media used. The interactive effects between studied factors and their influence on callus color and texture are also discussed in this paper.**

***Keywords* – Citrus, Callogenesis, Explant, Friable, Phytohormones.**

I. Introduction

Citrus is one of the most important crops worldwide. In Morocco, which is ranked 8th internationally in terms of fresh citrus fruit exportation [1], citriculture occupies a prominent place in the socio-economic activity. However, the development of this sector is still limited due to many abiotic (salinity, calcareous soils, drought) and biotic constraints, among which, the recent spread of Tristeza virus represents a serious threat.

Currently, most national research programs are focused on finding suitable rootstocks to replace sour orange, which is known to be highly susceptible to this disease.

Conventional breeding methods are usually limited due to nucellar polyembryony, high heterozygoty and long juvenile stage [2]-[3]-[4]. Recently, biotechnological progress has provided new approaches for genetic improvement of citrus [2]-[3]-[5]. *In vitro* multiplication, for example, as opposed to traditional regeneration, is faster, cheaper, more reliable and is therefore privileged in the case of international exchange of genetic material [2]-[6]. In addition, the use of tissue culture techniques proved to be efficient in producing healthy plants [2], [5]. As a result, protocols of tissue culture and micropropagation were generalized and thoroughly described for many citrus species, using a variety of explants [2], [7], [8].

Callogenesis is an important part of micropropagation process which consists in the ability to induce anarchical proliferation of parenchymal cells, leading to callus development [9]. It is a prerequisite for the initiation of somatic embryogenesis and therefore has to be well controlled. According to literature, the success of this step depends on different factors, which can be divided into two groups: (i) internal factors that are related to the plant such as the genotype, the developmental stage and the nature of the explant [3]-[10]-[11], and (ii) external factors which include hormonal composition of the culture medium, temperature, light, humidity and which were reported to influence considerably cellular proliferation during callogenesis and somatic embryogenesis in numerous plant species [3]-[10]. In our study, we used plant hormones, such as Kinetin, 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine that stimulate callus induction from plant explants [2]-[5]-[6]-[12]-[13]-[14]-[15]-[16].

The aim of this work is mainly to study the effects of some factors on the ability to induce friable calluses from citrus explants. These include the explant, the genotype and hormonal composition of induction medium, as well as the effects that may result from interactions between these factors. We particularly intend to provide a valuable database that can pave the way for somatic embryogenesis and sanitation works in citrus.

II. Materials And Methods

Plant Material

Flowers of 8 citrus genotypes, Amblycarpa (*C. Amblycarpa (Hassk.*) (Ochse), Cleopatra mandarin *(C.reticulata blanco)*, Carrizo citrange (*Citrus sinensis (L.) Osbeck X Poncirus trifoliata (L.) Raf.*), Hamlin orange

(Citrus sinensis (L.) Osb.), Rangpur lime *(Citrus x limonia Osbeck),* trifoliate orange *(Poncirus. trifoliata),* Troyer citrange *(C. sinensis X P. trifoliata*) and Volkamer lemon *(Citrus Volkameriana)* were collected from El Menzeh Experimental station (INRA, Kenitra) before anthesis to be used in this experiment.

Sterilization and Explant Preparation

Collected flowers were soaked for 3 min in 70% ethanol, immersed in 2% sodium hypochlorite for 20 min then rinsed 3 times using sterile distilled water. In a laminar flow hood under aseptic conditions, styles, stigmas and ovaries were cut and ovules were isolated using a binocular microscope.

Callus Induction

To induce callus development, the explants (styles and stigmas) were transferred to Petri dishes containing induction medium, which was composed of MS medium nutrients (Murashige and Skoog, 1969), supplemented with 500 $mg.l^{-1}$ malt extract, 40 $g.l^{-1}$ saccharose and different concentrations of phytohormones following the combinations shown in table 1. Concerning ovule explants, we chose MT medium (Murashige and Tucker, 1969) as a basic medium (Table.2) since it proved to favor callogenesis and ovule embryogenic capacity in our earlier works (data not published).

Tab. 1. Hormonal composition of induction mediums used in the case of style and stigma explants

Mediums	Hormonal composition
M1	_____
M2	BAP (0,5mg/l) + 2-4D(1mg/l)
M3	Kin (1mg/l)
M4	BAP (1 mg/l)

Tab. 2. Hormonal composition of induction mediums used in the case of ovule explants

Mediums	Hormonal composition
M1	_____
M2	BAP (1 mg/l)
M3	Kin (1 mg/l)
M4	Kin (2 mg/l)

Cultivation

In a laminar flow hood, the explants were placed in Petri dishes containing the induction mediums described above, and then kept in dark growth chambers at a temperature of 25 ± 2°C and a relative humidity of 70%.

Evaluation of Callus Induction

The ability to induce calluses was evaluated in the different explants by estimating the percentage of callogenesis. 4 repetitions (Petri dishes) were used for styles and stigmas with 5 explants per repetition, while 3 repetitions were used for ovule explants with 20 ovules per repetition.

Statistical Analyzes

Statistical analyzes were performed by ANOVA method using SAS package (version 9.0). Duncan's multiple range tests was also used at 5% significance level for post hoc comparison.

III. RESULTS

Callus induction started after 10 to 14 days of dark incubation. First, the explants (styles, stigmas and ovules) swelled due to internal cell divisions resulting in the appearance of small cell clusters at section zones (stigma and style) and explants sides which are in contact with the medium. These clusters generalized thereafter until covering the whole explants. (fig 1).

At the end of the experimental period, our observations of calluses derived from style and stigma explants revealed that these showed relative variations in terms of color and texture (case of style explants) as described in the tables 3and 4.

ANOVA results (Tab. 5) has also shown a significant variation of the percentage of callogenesis depending on the genotype used, the nature of the explant, hormonal composition of culture medium and the interactions between these factors.

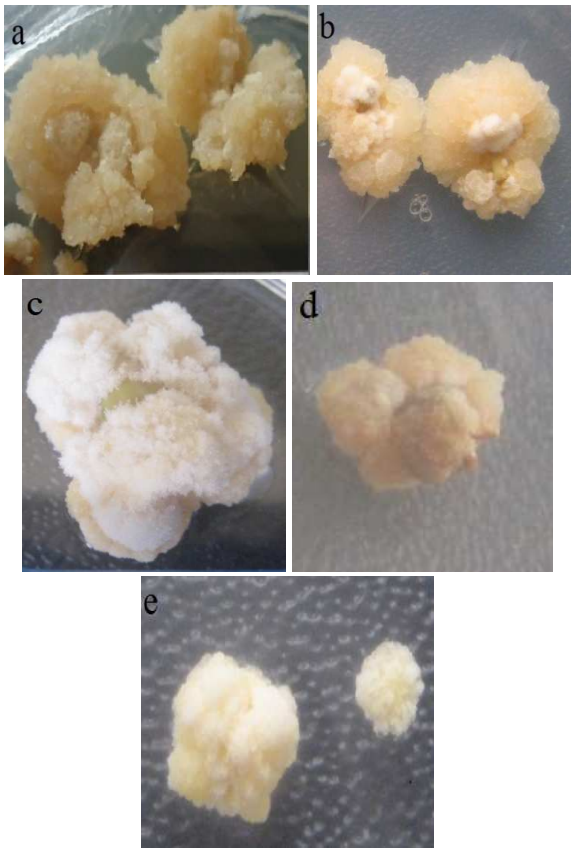

Fig. 1. Changes in morphology of calluses observed at the end of the experiment obained by culivated different explants on the medium M2. (a) and (b) represent a yellowish friable callus acquired from style explant taken from Carrizo (a) and Troyer (b) citranges, (c) represent a witish callus getting from style of Rangpure lime, (d) represent a yellowish compact callus obtained from stigma of Hamlin Orange, (e) illustrate a witish calluses gained from ovules of Carrizo citrange on the medium M4.

Tab 3. Variations in color and texture of callus obtained from eight styles of citrus genotypes tested on four culture media tested

Genotype	Color / Texture			
	M1	**M2**	**M3**	**M4**
Volkamer lemon	Brownish Compact	Yellowish compact	Whitish compact	Yellowish Compact
Amblycarpa	Yellowish Compact	Whitish compact	Yellowish compact	Whitish Compact
Trifoliate orange	_	_	_	_
Lime Rangpur	Yellowish Compact	Whitish compact	Whitish compact	Whitish Compact
Cleopatra mandarin	Brownish Compact	Yellowish compact	Brownish compact	Brownish Compact
Troyer citrange	_	Yellowish friable	Whitish compact	_
Carrizo citrange	Yellowish Compact	Yellowish friable	Yellowish compact	Whitish Compact
Hamlin Orange	Whitish compact	Whitish compact	Whitish compact	Whitish Compact

Tab. 4. Variations in color and texture of callus obtained from eight stigmas of citrus genotypes tested on four culture media tested

Genotype	Color / Texture			
	M1	**M2**	**M3**	**M4**
Volkamer lemon	Brownish Compact	Yellowish compact	Yellowish compact	Yellowish Compact
Amblycarpa	_	_	Yellowish Compact	_
Trifoliate orange	_	_	_	_
Lime Rangpur	Yellowish Compact	Whitish compact	Yellowish compact	Yellowish Compact
Cleopatra mandarin	Brownish Compact	Yellowish compact	_	Brownish Compact
Troyer citrange	_	_	_	_
Carrizo citrange	_	_	_	_
Hamlin Orange	Whitish compact	Yellowish compact	Whitish compact	Yellowish Compact

Tab 5. Effects of the different factors and interactions studied on percent callogenesis as resulted by ANOVA

Source	df	SS	MS	F value	Pr > F
Explant	2	85311,3712	42655,6856	110,55	< 0.0001
Medium	6	23372,6563	3895,4427	10,10	< 0.0001
Explant x Medium	3	7706,1250	2568,7083	6,66	0.0002
Genotype	7	114396,4063	16342,3438	42,35	< 0.0001
Explant x Genotype	14	140490,6250	10035,0446	26,01	< 0.0001
Medium x Genotype	42	51501,2604	1226,2205	3,18	< 0.0001
Explant x Medium x Genotype	21	34864,0000	1660,1905	4,30	< 0.0001

Callus Induction from Style and Stigma Explants

Effect of the Genotype

According to the results shown in table 5, the percentage of callogenesis is highly affected by the genotype ($P < 0.05$). Furthermore, the figures 2 and 3 show that, regardless of hormone combinations and their concentrations, or explant type, the rate of callogenesis was found to be higher in Hamlin orange, Volkamer lemon and Rangpur lime as compared to the other genotypes used. On the other hand, style and stigma explants of trifoliate orange were unable to produce calluses whatever the medium used. We should also note that the genotype affected considerably the color and texture of calluses derived from styles and stigmas as shown in tables 3 and 4.

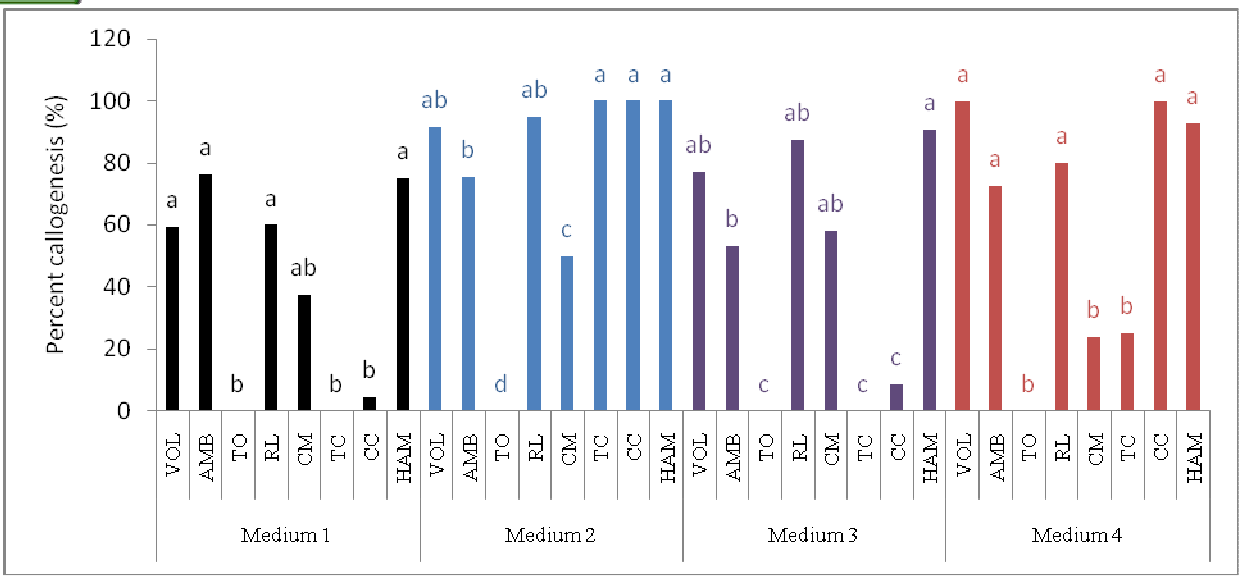

Fig. 2. Variations in percent callogenesis among 8 citrus genotypes when using styles as explants. (The letters indicate mean separation between genotypes as resulted by Duncan's multiple range test)

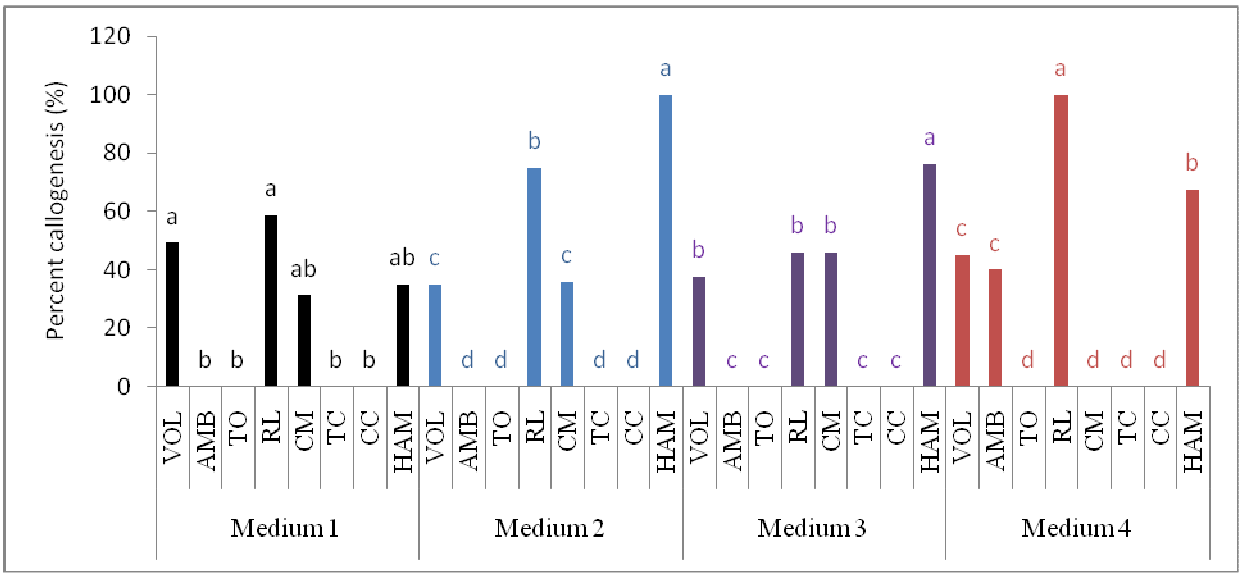

Fig. 3. Variations in percent callogenesis among 8 citrus genotypes when using stigmas as explants. (The letters indicate mean separation between genotypes as resulted by Duncan's multiple range test)

Effect of Hormonal Composition of Induction Medium

Growth regulators included in the composition of induction mediums exerted a significant effect on percent. In most cases, these resulted in a stimulation of callus induction compared to control medium (M1) which is hormone-free (fig. 4 and 5). The medium M2 including a combination of 2,4-D (1 $mg.l^{-1}$), which an auxin, and BAP (0.5 $mg.l^{-1}$), which is cytokinin resulted in the highest callogenesis rates, particularly for styles of Carrizo and Troyer citranges (100% callogenesis).

Moreover, calluses obtained under these conditions were characterized by a yellowish color and a friable texture. On the other hand, mediums containing one cytokinin improved slightly callogenesis ability of the explants. However, these resulted in compact calluses and the percentage of callogenesis depended on the nature of the cytokinin used. Indeed, results recorded on the medium M4, containing BAP at 1 $mg.l^{-1}$ were higher than those observed on the medium M3 containing Kinetin at the same concentration (tab. 1).

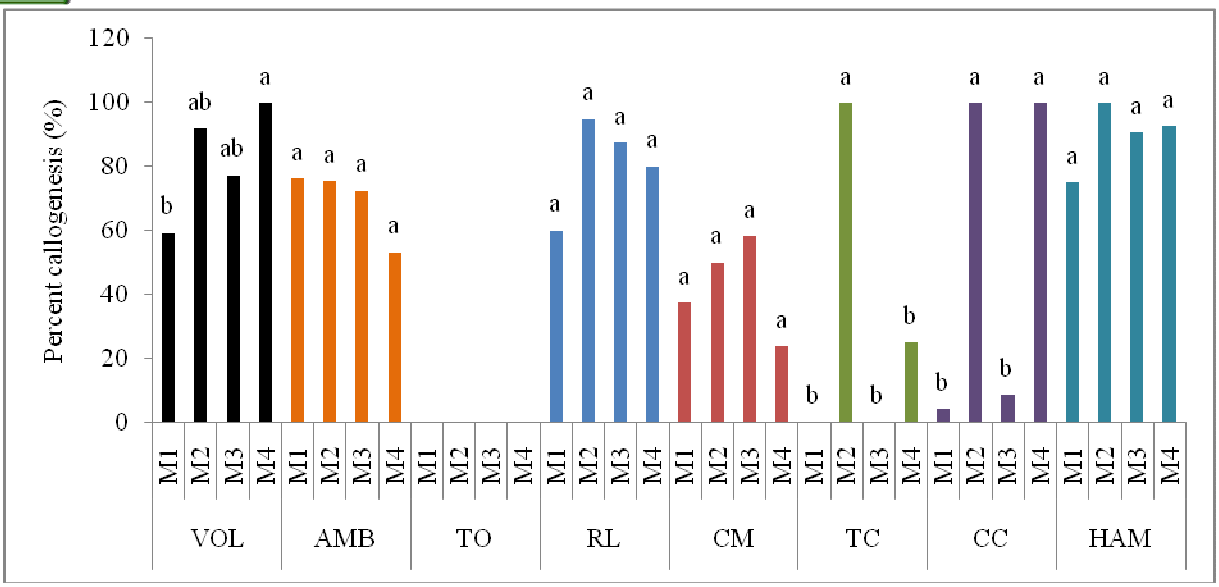

Fig. 4. Variations in callogenesis among 4 induction mediums when using styles as explants. (The letters indicate mean separation between mediums as resulted by Duncan's multiple range test)

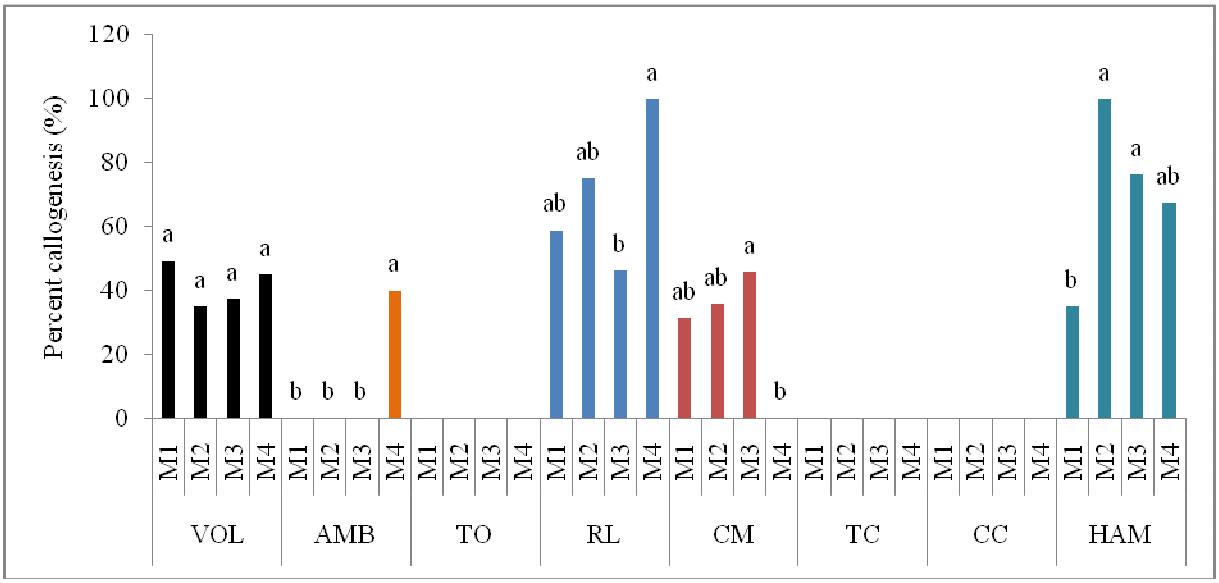

Fig. 5. Variations in callogenesis among 4 induction mediums when using stigmas as explants. (The letters indicate mean separation between mediums as resulted by Duncan's multiple range test)

Effect of the Explant

Statistical analyzes showed significant differences (P<0.05) in percent callogenesis not only among genotypes, but also among somatic tissues used as explants in this study (Tab. 5). Compared to style explants, stigma explants gave higher values in all the mediums tested. However, except for Rangpur lime, Hamlin orange and Volkamer lemon, stigma explants were unable to produce calluses. Notably, those of Amblycarpa were only successful when cultivated on the medium M4, which is supplemented with 1 mg.l^{-1} BAP.

The color and the texture of calluses were also traits that varied considerably with time and depending on the nature of explant. Indeed, we observed that some calluses derived from style and incubated in the dark for 2 months had a compact texture, but became gradually friable afterwards (4 months). This observation is valid for Carrizo and Troyer citranges (fig. 1a and 1b).

Callus Induction from Ovule Explants

Similar to styles and stigmas, there were significant variations in percent callogenesis (P<0.05) from ovule explants depending on the genotype and the hormonal composition of induction medium. In general, maximum values were recorded on the medium M3, which is MT medium supplemented with 1 mg. l^{-1} Kinetin, followed respectively by M1, M2 and M4 (fig. 6). However, poor results were obtained in ovules of Hamlin orange using the same medium (fig. 7). Regarding callus morphology, all genotypes resulted in a whitish color and a compact texture under these conditions (fig. 1e).

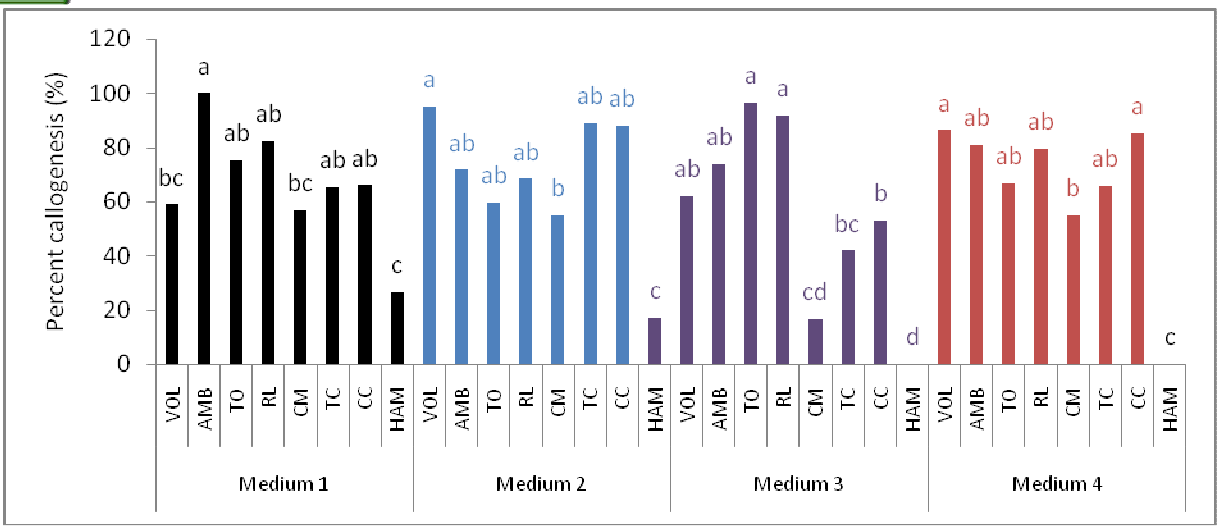

Fig. 6. Variations in percent callogenesis among 8 citrus genotypes when using ovule as explant. (The letters indicate mean separation between genotypes as resulted by Duncan's multiple range test)

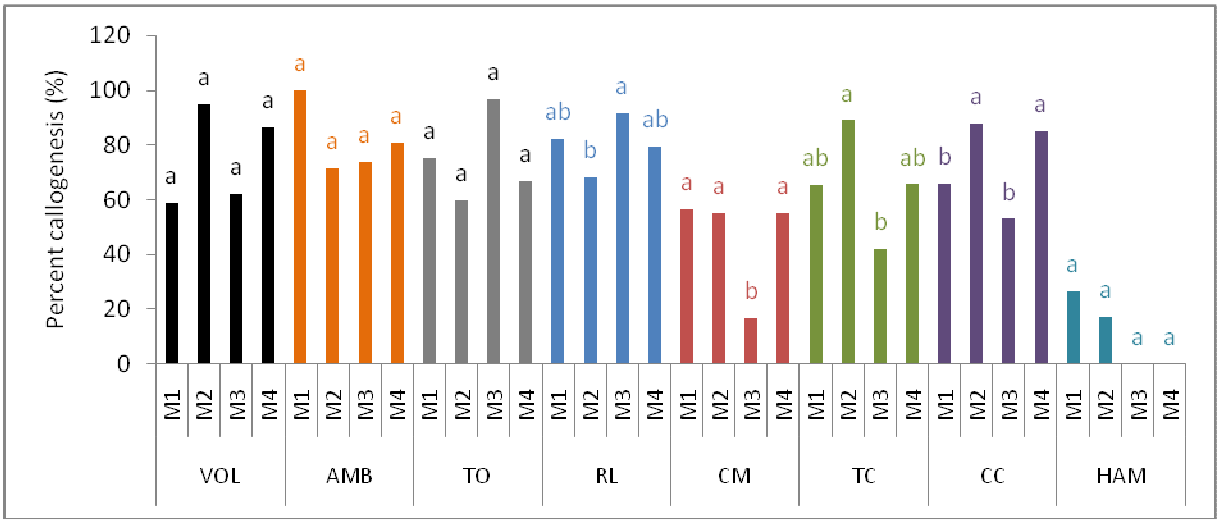

Fig. 7. Variations in percent callogenesis among 4 induction mediums when using ovule as explant. (The letters indicate mean separation between mediums as resulted by Duncan's multiple range test)

IV. Discussion

In this work, we tested the effect of three important factors on callogenesis ability of different citrus varieties: the genotype, the nature of the explant and hormonal composition of culture medium. The same work was reported earlier in *Scopiurus* [10] and chickpea (*Cicer arietinum* L.) [15].

Our results have shown that the use of a hormone-free MS medium gives lower percentages of callogenesis than mediums supplemented with hormones, which indicates the effectiveness of these compounds in promoting callus induction. These results are in line with those reported by [17] and [18] Concerning style and stigma explants, our results have shown that maximum percentage of callogenesis was observed under the combination 1 $mg.l^{-1}$ 2,4-D and 0.5 $mg.l^{-1}$ BAP, which resulted furthermore in a friable texture of calluses. Similar findings were reported in citrus by [3]-[10], and [15] in chickpea. Also, using the same hormone combination, obtained satisfactory results in *Scorpiurus*. However, [19] obtained compact calluses in an attempt to induce embryogenic calluses from wheat zygotic embryos.

A review of literature shows that BAP is more effective than Kinetin in promoting callogenesis. This fact was either confirmed in citrus [6] and other plant species such as Gerbera (*Gerbera jamesonii Bolus*) [18] Calluses obtained in presence of BAP were described to be compact, of whitish color and having a successful growth [18]. In our case, the results recorded on the medium M4, containing BAP at 1 $mg.l^{-1}$ were better than those observed on the medium M3 containing Kinetin at the same concentration.

In addition to hormonal effects, the nature of explant was found to affect significantly the percentage of callogenesis as well as callus morphology. When using MT as a basic induction medium, calluses were found to develop easily from ovule explants as suggested by high callogenesis rates. However, these tended to have a compact texture and a whitish color. Similar observations were reported by [7]. Concerning MT basic medium, callogenesis from non fertilized ovules reached 100%,

which is contrasted with the findings of [11]. These authors studied the effect of sampling date and reported that ovules collected 5 weeks before anthesis are more suitable for callogenesis than ovules collected 3 weeks before anthesis. This fact, in addition to genotypic variations, may explain the differences between callogenesis-related studies. According to [11], it is likely that endogenous hormone balances of ovules are variable depending on genotype and environmental condition, which make it more complicated to draw reliable conclusions regarding the suitable period for collecting explants in order to induce callogenesis.

Another relevant finding of the present investigation is the clear genotypic differences in response to medium and explant variations with respect to callus induction. Indeed, some cultivars showed a recalcitrant behavior under specific conditions, such as trifoliate orange in the case of style and stigma explants. Other genotypes manifested generally a poor callus induction ability, especially when using stigmas as explants. These included Amblycarpa, Rangpur lime and Carrizo and Troyer citranges. A third group of genotypes gave satisfactory results on most of mediums studied. This was the case of Rangpur lime, Hamlin orange, Amblycarpa, Volkamer lemon and Cleopatra mandarin for style explants, and Rangpur lime and Hamlin orange for stigma explants. The involvement of genotypic factor in the success of callogenesis was previously described in citrus ovary [7] and ovule explants [11] and in *Scorpiurus* explants [10]. Furthermore, the study of [11] have shown that differences among genotypes are correlated with *in vivo* polyembryony level of the cultivars and suggested that these differences may be due to variations in endogenous hormone balances among genotypes.

V. Conclusion

Conditions for initiation in citrus were optimized in this study; Callogenesis showed range of responses depending on medium formulation, growth regulators combinations and concentrations, genotype and explant.

It has resulted that MS medium supplemented with 1 $mg.l^{-1}$ 2,4-D and 0.5 $mg.l^{-1}$ BAP, was benefic to induce friable callus, also the callogenesis from style was optimal than from stigma and ovule explants. These results will help to pave the way to works related to somatic embryogenesis in citrus.

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