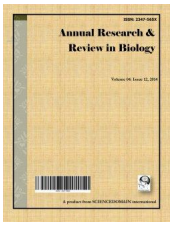

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Plantlet Regeneration from Unfertilized Ovule of Mandarin (*Citrus reticulata*)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript

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ABSTRACT

The genus Citrus contains numerous fresh and processed fruit cultivars that are economically important worldwide, many genotypes are amenable to somatic embryogenesis, which became a key regeneration pathway in many experimental approaches of cultivar improvement. in this objective We aime at studying the effects of various culture media on the induction and the development of citrus somatic embryos.Callus cultures were initiated from the infertlized ovules of six varieties of mandarin (Anana, Lee, Murcott, Ortanique, Temple, and Wilking) within 3 media: MT (Murashig and Tuker, 1969) without hormones, MT + 1 mg/l BAP, MT + 1 mg/l Kinetin, the experiments show a highly significant effect ($P < 0.001$) of the culture media and genotype. No reactivity was observed on the MT environment in the absence of growth regulator, while the culture media MT in addition to 1 mg/l BAP gave the best results of induction of embryogenic callus induction. The induction of somatic embryogenesis was obtained on MT media without hormones. For the plantlets regeneration the favorable media was MT without hormones or added to ANA and active coal.

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1. INTRODUCTION

The genus Citrus, which actually makes one of the most important fruits worldwide, belongs to the family Rutaceae, which comprises 140 genera and 1300 species throughout the world [1]. C. sinensis is one of the major commercial fruit crops that is largely consumed both as fresh fruit or juice due to the high amount of vitamin C it contains and its antioxidant value [2]. It should be mentioned that genus citrus is among Morocco's top agricultural exports. In this regard, its agriculture needs special consideration in terms of culture techniques to cater for the diversified needs of consumers and crop breeders. However, genetic manipulation through conventional methods remains a challengingt task for farmers; genus Citrus provides various biological limitations that comprise long juvenile period, high heterozygosity, sexual incompatibility, nucellar polyembryony and large plant size that greatly hinder cultivar improvement [3].

Advances in biotechnology have generated new opportunities for better citrus genetic cultivation. In vitropropagation is one potential technique that can help develop problems related to the field culture for suchspecies [4]; besides,Techniques like *in vitro* culture made it easy to improve citrus against different biotic stresses. Low yield can preserve important citrus genotypes through somaclonal variations [5] somatic cell hybridization [6,7]. in fact ,transformation of high yielding cultivars [8] has proven effective in promoting disease free plants. However all these highly sophisticated techniques require the presence of a highly responsive regeneration protocol.

The development of efficient plant tissue culture procedures for embryogenic culture induction, maintenance and plant regeneration in Citrus is important for the application of different technologies for crop improvement. Several citrus species have been regenerated by somatic embryogenesis from explants derived from different parts of the plant. Somatic embryogenesis has been obtained in Citrus through the culture of entire fertilized ovules, excised nucelli from fertilized ovules, or isolated nucellar embryos from polyembryonic Citrus genotypes [8]. Somatic embryos have also been produced from abortive [9], unfertilized [10] and undeveloped ovules [11,12].

The factors that can influence callogenesis and somatic embryogenesis are multiple. The callogenic and embryogenic potentialities appear to be strongly different depending on the genotype [13,12,14]. This difference in reactivity is often correlated with the level of in vivo polyembryony [12]. and could be explained by different endogenous hormonal balances. Thus, Tisserate and Murashige [15]. showed that the presence of ovules of Cedarwood (monoembryony) inhibited Somatic embryogenesis in Ponkan mandarin (polyembryonic) callus placed in the same culture dish Mitra and Chaturvedi [16]. have reported a direct correlation between the degree of polyembryony *in vivo* and the attitude to regenerate somatic embryos *in vitro* Polyembryony is the most beneficial and distinct character in citrus seeds. This characteristic can be beneficial in citrus improvement programs.

Production of callus and its subsequent regeneration are the prime steps in crop plants to be manipulated by biotechnological means and to exploit somaclonal variations[17]. The composition of culture medium and culture conditions have been shown to be crucial for the growth of in vitro culture [18].

The study presented in this article is part of the program of improvement of citrus varieties launched by INRA Morocco targeting two main objectives which are the increase of the variabilitygenetics via callogenesis and somatic embryogenesis,and to investigate the influence of explant and of culture media on induction of embryogenic cultures and embryo development to explore efficient procedures to induce somatic embryogenesis from unferltilized ovules. in a mandarin [*C. reticulata*.].

2. MATERIALS AND METHODS

The plant material used was collected from field grown trees belonging to the National Institute of Agronomic Research in Kenitra. The work concerns 6 varieties of mandarin (*C. reticulata* L.). This species, genetically diversified, generally has good ability to somatic embryogenesis. the unfertilized ovules of six citrus genotypes were selected for this study: four late varieties (Murcott honney, Wilking, Ortanique, Anana) and two earlies varieties (Lee,

Temple), which also differs between them by the level of polyembryony (Table 1).

2.1 Disinfection and Preparation of the Explants

The ovaries have undergon a quick soak in ethanol 70% (v/v) for 10 min, followed by immersion for 20 min in calcium hypochlorite 2% (m/v) and washed three times with sterilized distilled water for 15 min, after the sterilization, the unfertilized ovules were excised with a scalpel and pliers, under a binocular microscope unfertilized ovules were isolated and placed in culture media. Twenty ovules placed on each petri dish. All these operations were conducted under a laminar flow hood.

2.2 Media and Culture Conditions

2.2.1 Embryogenic callus induction

For all experiments of callus induction, the basal media used was described by Murashige and Tucker (1969), Containing 50 g/l sucrose and 0.5 g/l of extract of malt and pH adjusted to 5.7. Three medias were tested, as M0 was devoid of hormone, M1 was supplemented with 1 mg/l BAP (6-benzylaminopurine) and M2 supplemented with 1 mg/l Kinetin, (Table 2).All prepared culture were sterilized by autoclaving at 121ºC under a pressure of 1 bar for 30 minutes. Then, they were placed in a growth chamber in the dark at a temperature of 26 ± 1ºC distributed under aseptic conditions.

2.3 Seedling Regeneration and Root Proliferation

The primary callus was divided and incubated on the same media to stimulate embryogenesis callus . The embryogenic callus obtained from the previous step was incubated on MT media. Somatic embryos were obtained after 4 weeks of culture.The fully developed somatic embryos were cultured, each in a test tube containing MT media. The three media tested were as follows: M3 (MT) , M4 (MT + 1 mg / l ANA) and M5 (MT + 1mg /l ANA 4g/l active coal) . Fully developed plantlets were obtained.

2.4 Acclimatization

Rooted plants went through a hardening phase in preparation for the acclimatization stage. This phase aims at opening the test tubes, cleaning the roots which contain the culture media solidified with agar, and closing these tubes with cotton. This stage lasted about thirty hours at room temperature. Each plantlet was then transplanted into a pot containing a mixture of sterilized sand and soil (1:1, v/v) and placed under greenhouse at a temperature of 26 ± 1ºC and a relative humidity of 80%. The pots were covered with a para film from three weeks to a month to maintain high humidity around the young seedling.

Table 1. List of mandarin varieties used

	Varieties of mandarin		
	Name	**Parents**	**level of polyembryony**
Late variety	Ortanique	C. sinensis X tangerine	Polyembryonic
	Anana	C. reticulata Blanco	Polyembryonic
	Murcott (Tangor)	C. reticulata X C. Sinensis	Polyembryonic
	Wilking	C. nobilis X C. deliciosa	Monoembryonic
Early variety	Temple (Tangor)	C. reticulata X C. sinensis	Monoembryonic
	Lee	C. paradisi X tangerine	Monoembryonic

Table 2. Composition of the tested culture media

	Media	**Composition**
Embryogenic callus induction	M0	MT
	M1	MT+1mg/l BAP
	M2	MT+1mg/l Kinetin
Plantlet Regeneration	M3	MT
	M4	MT + 1 mg ANA
	M5	MT + 1mg /l ANA+4g/l active coal.

MT : Murashige and Tucker

3. RESULTS AND DISCUSSION

Callus induction was generally carried out in ovules citrus fruit [19]. The initiation of calluses in the different varieties of mandarin was affected by the variability of the cultivars and by the culture media, The results obtained show that the ability of callogenesis and somatic embryogenesis differs not only according to the culture media but also according to the varieties used. This was deduced from the variance analysis which showed a highly significant effect of both factors varieties (V) and culture media(M), and a significant effect of the interaction culture media variety (VxM) (Table 3).

The Statistical analysis results presented here demonstrate a highly significant effect of varieties($P<0.0001$), the percentage of calluses production varied from 22% to 60%. The Murcott and Anana mandarins showed the highest callus induction rate with 60% for each one, followed by the variety of Temple with (50%), then Wilking (35%), Ortanique (30%) and Lee (22%) (Fig. 1).

During the callus induction, about 4 to 20 days after the incubation , most of varieties produced callus. The difference of callus induction period is influenced by two factors; namely: hormonal composition of culture media and the varieties (Table 4). The culture media M1 containing the BAP represents the longest duration (15 to 20 days), then M2 (4-15 days). So, the responses of the callus formation differ according to the varieties: 5-6 days for the ortanique with the fastest initiation within two culture media, followed by the variety Anana whose response is from 8 to 10 days. For Temple, Murcott and Lee, the response is between 10 and 15 days.

Environment, conditions of growth, genotype, age of explants, components of culture media are factors that strongly influence the induction of embryonic callus cells [20]. The variance analysis showed that the frequency of callus induction was significantly affected by media, basd on the results obtained, MT medium supplemented with BAP (M1) showed a greated potential reponse in terms of pourcentage of callus induction (Fig. 2), As already observed in the Citrus from ovules culture [21]. and style [22]. While M2 media promoted the induction of callogenesis and embryogenesis of 24 % and 62%, Starrantino and Russo [23] reported that somatic embryogenesis from ovules culture can induce embryogenesis in the range of 0-70% depending on the genotype, and this confirms the results obtained.

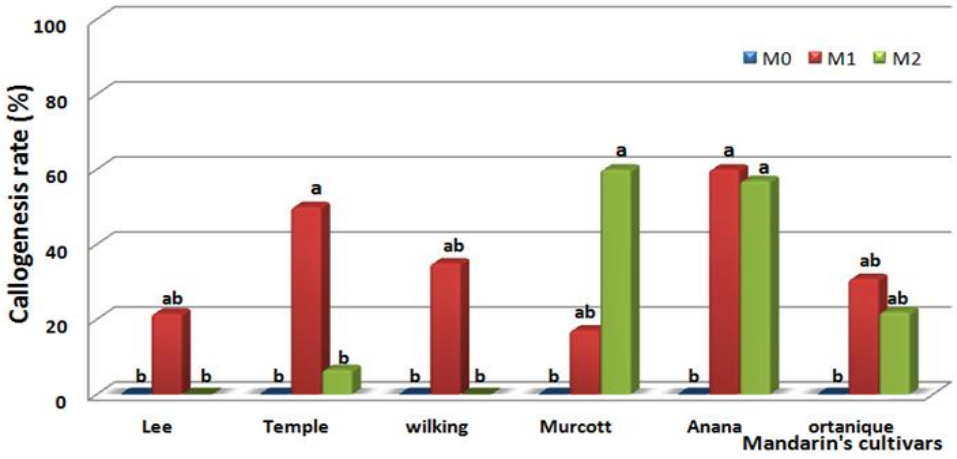

Fig. 1. Callus rate according to the varieties of mandarin and the media used
(Varieties followed by the same letters are not significantly different to the threshold 5%(Test Duncan).

Table 3. Result of variance analysis of callus rate

Source	DF	Type I SS	Mean square	F value	Pr > F
Variety (V)	5	25773.90111	5154.78022	7.45	<0.0001
Media (M)	3	29772.58675	9924.19558	14.33	<0.0001
(V) X (M)	10	24090.68800	2409.06880	3.48	<0.0005

Table 4. Duration of callus induction in the mandarin varieties

Varieties	Callus induction duration (Days)	
	M1 MT+1mg/l BAP	M2 MT+1mg/l Kinetin
Murcott	15	10
Anana	8	10
Lee	15	-
Wilking	20	-
Ortanique	6	5
Temple	10	15
Average	12	10

Qualitative evaluations were conducted after culture, and callus were classified into categories, based on their texture and colour, which varied between white, yellow and brown colour, or friable translucent and compact texture which varied between white yellow and brown-coloured, or Friable,Translucent and compact texture .The composition of growth regulators had great influence on the colour and texture of callus. The MT medium containing BAP (1.0 mg/l) seems to stimulate the reactivity of the ovules by the induction of embryogenic callus in different varieties of mandarin, (Table 5, Fig. 3).

Calluses that were generated from unfertlizied ovules, an indirect somatic embryos. Somatic embryos were green and easy to detach (Fig. 3). Different embryo stages (globular, heart-shaped, torpedo and cotyledon stage) were observed .Embryos differentiated on the surface of the cals were then transferred into a test tube containing the culture devoid of hormone M3 media, then M4 containing 1 mg/l ANA and M5 1 mg/l Anana + 4 g/l of active coal. The rate of plantlets regeneration varied according to the culture media and the variability of cultivars, the highest regeneration rate was marked in the Anana variety with 82%, followed by the murcott 72%, Temple 45% and ortanique 33% (Fig. 4), The development of embryos on the three tested media (M3, M and M5) was favorable to obtain plantlets in the presence of auxin which favors regeneration of seedlings and active coal - promoted rooting (Fig. 5).Generated diploid plantlets were grown in tube for 3 months before the acclimatization phase in vivo.

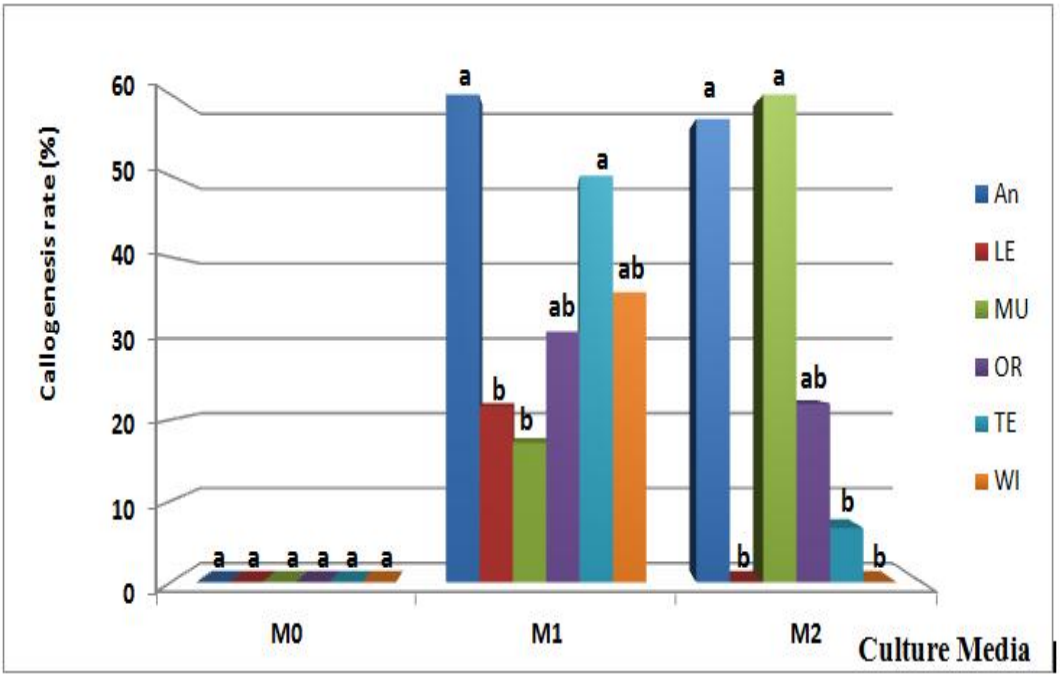

Fig. 2. Effect of the cultural media on the callus induction and somatic embryogenesis (M0:MT;M1:MT+1mg/lBAP;M2:MT+1mg/lKinetin).An:Anana,LE:Lee,MU:Murcott,OR:Ortanique,TE:Temple,WI:Wilking

(culture media followed by the same letters are not significantly different to the threshold 5%(Test Duncan).

Table 5.Texture, appearance and coloring of callus resulting from the induction from the mandarin ovules

Varieties	Supplement (mg/l)	Ovary that cultured	Callus response (%)	Colour of callus	Type of callus
Murcott	1mg/l BAP (M1)	20	17 58.3	Brown Yellowish	Friable Translucent Compact
	1mg/l Kinetin (M2)	20	60 40	Brown Yellowish	Friable Translucent Compact
Anana	1mg/l BAP (M1)	20	60 40	Whitish Yellowish	Friable,Translucent Compact
	1mg/l Kinetin (M2)	20 20	57 43	Whitish Yellowish	Friable Translucent Compact
Lee	1mg/l BAP (M1)	20	21 40	Brown Yellowish	Friable Translucent Compact
	1mg/l Kinetin (M2)	20	100	Brownish	Compact
Wilking	1mg/l BAP (M1)	20	35 55	Yellowish Yellowish	Friable Translucent Compact
	1mg/l Kinetin (M2)	20	100	Yellowish	Compact
Ortanique	1mg/l BAP (M1)	20	30 69	Brownish, Yellowish	Friable Translucent Compact
	1mg/l Kinetin (M2)	20	22 78	Brownish Yellowish	Compact Compact
Temple	1mg/l BAP (M1)	20	50 50	Brown Yellowish	Friable Translucent Compact
	1mg/l Kinetin (M2)	20	6.6 93.3	Brown Yellowish	Friable Compact

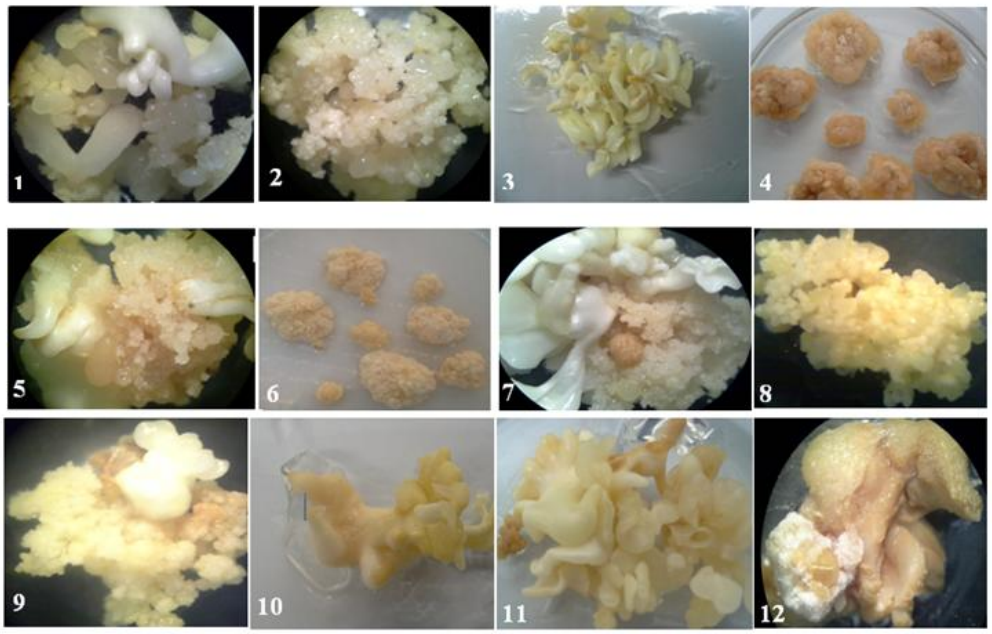

Fig. 3. Callus induction from unpollinated ovary culture :1 :Anana (M1) ;2 :Anana (M2) ; 3 :Lee (M1) ;4 :Lee (M2);5 :wilking (M1) ;6 :wilking (M2) ;7 : Murcott (M1);8 :Murcott(M2) ; 9:Temple(M1);10:Temple(M2) ;11 :Ortanique (M1);12 : Ortanique(M1)

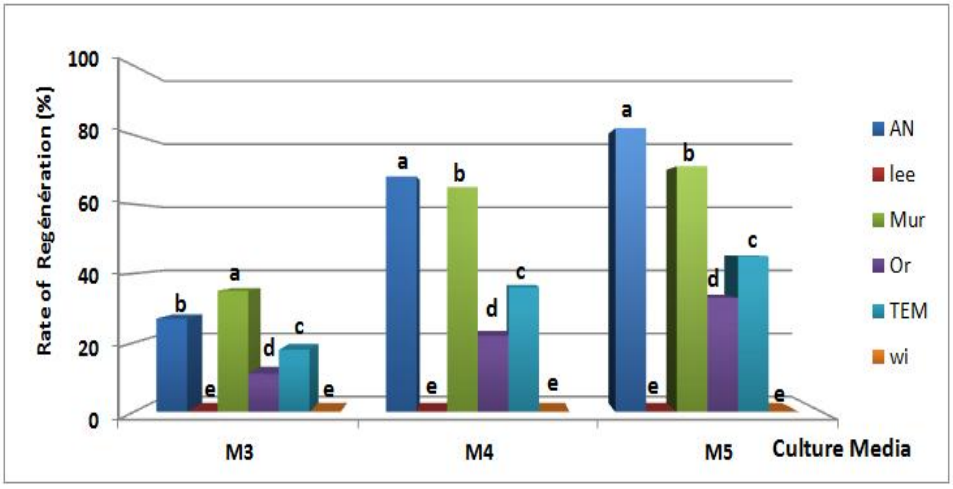

Fig. 4. Percentage of plantlets regenerated in mandarin varieties of M3 (MT),M4 (MT+1 mg/l ANA),M5(MT+1mg /l ANA+4g/l active coal)

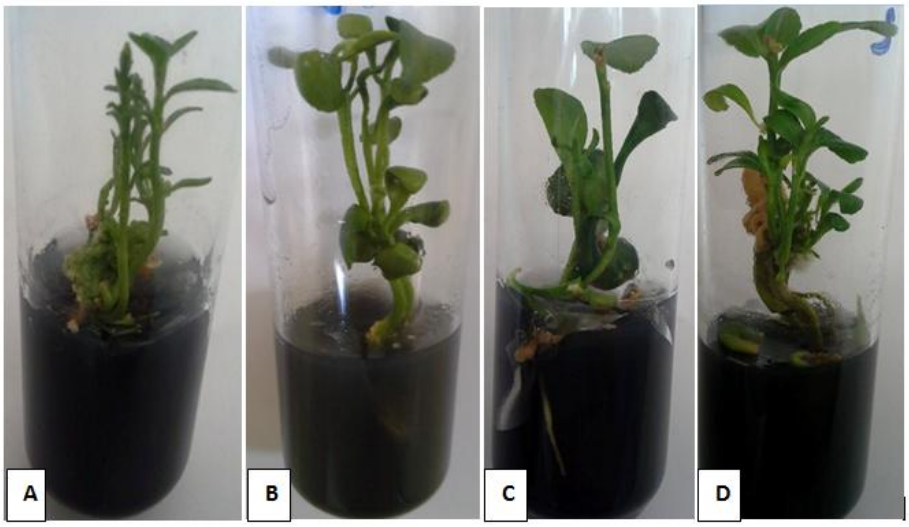

Fig. 5. Seedling obtained from embryogenic callus, A: *Temple,* B: *Anana;* C: *Murcott;* D: Ortanique.

We tried various concentrations and combinations of hormones in six varieties of mandarins (Citrus Reticulata) for callus induction to identify the ideal conditions for the developpement of embryogenic callus and plantlet regeneration. About 4 to 20 days after the incubation, most of different genotypes produced a friable creamy-white calluses. The variable proliferation rates of callus production were observed during the callus induction period. Statistical analysis revealed that the frequency of callus induction was highly affected by media culture and genotype. Indeed, the percentage of calluses production varied from 22% to 60%. Among Citrus species, Murcott and Anana showed the highest callus induction rate that are both 60 in M2 medium wich contain 1 mg of kinetin , The MT medium supplemented with BAP proved to be the best medium for callus induction for all types of varieties . Benzyl adenine for the induction of somatic embryo has also been reported by Praveen et al (2003) [24] for Kinnow mandarin, Contrary to Komamine and Fujimura [25], who observed that BAP and kinetin were inhibitors of somatic embryogenesis in carrots. Our results confirm those of yasuda et al [26], which confirmed that embryogenesis could be induced in coffee in the presence of a

single cytokinin [27], raised the issue of the embryonic potential which could be increased by using different plant growth regulators. Cytokinin is a plant hormone that controls the growth and development of plants, originally identified as a factor that induces cell division in the presence of auxin [28]. Subsequently, Skoog and Miller [29] observed that cytokinins were antagonistic to the auxin, the remarkable effect of cytokinin suggests that cytokinin plays a central role in the regulation of cell division. The induction of callogenesis from ovules from the six varieties of mandarins that were the subject of this study , showed a highly significant effect of the two factors culture media and variety type as shown in date [30] . The three varieties Anana, Temple, and Murcott showed percentage induction of embryogenic callus ranging between 60% and 50%, this confirms the results by Handaji et al [31] , followed by the variety, Ortanique of 30.8%, lee and wilking 21.6% And 35%, respectively. This difference in reactivity between cultivars has also been found by many authors such as Kochba [32], Gmitter [33] and Bugam [34]. According to Moore [12] it seems that besides the level of polyembryony other factors have an influence on embryogenic and organogenic capacity of embryonic callus. This difference of embryogenic response was not surprising, since Kobayashi et al. [6]; Mitra & Chaturvedi [16], found a direct relationship between the level of polyembryogenesis in a genotype and its potential for in vitro embryoid formation.The results reported here indicate an overall favorable effect of cytokinins on the frequency of callus emergence. In addition, kinetin would favour in certain genotypes the production of friable embryogenic callus from ovules culture (Fig 3). Such results had previously been demonstrated by [35,36]. The differences in callogenesis rates observed in the six genotypes for the same type of explant indicated that the genetic constitution is also an important factor affecting the induction efficiency of callus.Benedito et al [37] found different results on callus induction after culture of unfertilized ovules from six soft orange cultivars. For the regeneration of the seedlings, the three tested media (M3, M4 and M5) allowed to give rise to young plants with or without roots. Thus, auxin favored the regeneration of the seedlings and the activated coal has rather promoted rooting ,the result was compatible with those of Hmouni [38]. the frequency of the germinated embryos into plantlets was higher in Anana 82%, followed by the murcott 72%, Temple 45% and ortanique 33%, our results are similar to those of Paul [39], Beneditu [40], and Chandra [41] with their fellows, who observed a good percentage of shoot formation in the presence of auxin in culture media. Ling et al [42], also observed good root formation in the presence of auxins in culture media, Except that Lee and Wilking varieties did not both give rise to any seedlings, the absence of regeneration could be explained only by the monoembryony of these two varieties and this has been reported by Moore [43], who suggested that the seedling regeneration procedure does not work with monoembryonic genotypes. It's confirms the result obtained by Kobayashi et al [44], who reported that somatic embryogenesis from unfertilized ovules of monoembryonic genotypes were unsuccessful [45]. The monoembryonic cultivars apparently contains substances inhibitory to embryogenesis [46], they are likely to include high concentration of several endogenous growth regulators and other unkown factors [15], but there have been no accounts to date of successful attempts to this information this is proved by the results obtained in the Temple (monoembryonic) and which gave a regeneration rate of 45%. All the results obtained are similar to those by Button and Bornman [10], who found that plantlets have been produced by cultured ovules (fertilized and unfertilized) from both mono- and polyembryonic species.

In summary, this work represents the comparative study of the influence of the culture medium and types of varieties on the growth of callus.results shows that callus induction and plantlet regeneration are not affected only by growth regulators but also by the varieties even if all of them belong to the same group citrus reticulata, the genetic constitution of each variety is an important factor of the efficient induction.

4. CONCLUSION

Preliminary results of this investigation indicated that SE using unfertilized ovules culture has been successfully applied to regenerate different genotypes of the main Citrus species. The successful results of Callus formation rates and plantlet regeneration varied among the six varieties . Molecular analysis of regenerated plantlets will be studied by DNA to evaluate the level of somaclonal variation relative to the parent plant. Further studies need to be conducted to investigate the embryogenic potential of other citrus genotypes of high economic importance

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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