Determination of genetic fidelity of Algerian citrus genotypes regenerated by somatic embryogenesis .

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Abstract

The multiplication of 'healthy' propagating material is of utmost importance in the control of the most serious virus affecting citrus trees worldwide. All the sanitation methods available are effective in elimination of graft-transmissible pathogens .however somatic embryogenesis from stigma and style are the preferred ones for their numerous advantages.This method was applied in this study on Algerian Citrus collection . Different Citrus genotype used were infected, mainly by viroid agents . Most of the tested genotypes proved to regenerate somatic embryos in a different period of time somatic embryos were germinated and converted into plantlets. The mother plant and some regenerants, coming from different embryogenic events, were selected for the analyses of genetic stability by using 12 ISRR primers. The plants regenerated from Metidja Navel group genotype showed genetic polymorphic fragment for 8 primers, while in W. Navel genotypes, the mother and micropropagated plants showed the same profile only for ISSR12-12.

Keywords: ISSR analysis , Somaclonal variation, Somatic embryogenesis, stigmate1style , Citrus

Introduction

Somatic embryogenesis is the process through which somatic cells develop into plants through an orderly series of characteristic embryological stages without fusion of gametes [1]. Historically, somatic embryogenesis was described for the first time almost 50 years ago [2] and due to the importance shown, it has been the subject of many studies. In citrus,

somatic embryogenesis has been observed through the culture of entire fertilized ovules, or isolated nucellar embryos from polyembryonic citrus genotypes [3]. This technique involves the use of different explant types (thin cell layers of stigma and style or entire organs) excised from citrus flowers tissues, which are not ovular in origin and regenerate plantlets being genetically identical to the original source [4-5]. Somatic embryogenesis from styles and stigmas represents a recent technique for citrus sanitation and has been successfully practiced in the regeneration of different citrus genotypes [6].

Algeria has a citrus germplasm collection of 178 varieties, which represents genetic resources of inestimable value [7]. The objective of the present study is the application of style and stigma somatic embryogenesis (SE) for the regeneration of a number of citrus genotypes which are mainly maintained in the citrus collection, Algeria. The evaluation of the embryogenic potential of citrus genotypes, mainly of the native ones, will provide useful information in order to preserve citrus genotypes as healthy germplasm collection and introduce this technique in the Algerian establishing certification program of the citrus propagating material.

Materials and methods

Plant material

Flowers used in this work were collected from 25 genotypes of different citrus species of orange (*Citrus sinensis*), lemon (*C. limon*), mandarin (*C. reticulata*) and grapefruit (*C. paradise*) (Table 1). This material was obtained from the Algerian collection (with exception of 2 lemon genotypes coming from a private orchard. Flowers were collected before their opening from plants growing under field conditions, during the period of full bloom. They were surface-sterilized by immersion for 5 min in 70% ethanol, 20 min in 2% (w/v) sodium hypochlorite, followed by three 5 min rinses in sterile distilled water. Stigmas

and styles were excised with a scalpel and vertically plated as single explants into medium-sized Petri dishes (100X 15mm) with the cut surface in contact with the medium. Five explants were placed in each Petri dish and 6 Petri dishes were used per treatment.

Culture medium.

Styles and stigmas were cultured on MS solidified medium [8]. (8 g/l Phyto agar) with 500 mg/l malt extract and 146 mM sucrose as carbon source. The pH of the media was adjusted to 5.7±0.1 with 0.5 M of KOH before autoclaving. Explants were cultured in the presence of 3mg/l of 6-benzylaminopurine (BAP) (MS II). Hormone-free condition was used as control (MS I).

Explants and calluses were subcultured into fresh medium at 4–6 weeks intervals and maintained in a growth chamber at 25º±1ºC under a 16h daylength photoperiod.

Germinated embryos were isolated and transferred into test tubes (1 embryo per 55 X 23mm glass tube sealed with parafilm M) containing 20 ml of the above-mentioned hormone-free solid medium (MS I).

Genetic fidelity analysis of the regenerated plants.

DNA analysis (ISSR) were carried out to verify the genetic fidelity of the regenerated plants. A molecular markers analysis was conducted for 10 regenerants from each cultivar and compared with the respective mother plants. For this analysis 12 ISSR primers were used. PCR-reaction products were electrophoresed on a 1.5% (w/v) agarose gel. Only those bands showing consistent amplification were considered;.

Data and Statistical analysis

Explants of 4 citrus species were periodically observed to check when callus formation occurred and first embryogenic event took place. Percentage of calluses and embryogenic explants (styles and stigmas) were evaluated.

Statistical analysis was performed using percentages of callus and somatic embryo induction. The experimental design was completely randomized with 6 replicates of Petri dishes. Effects of treatment were statistically tested ($P=0.01$)

Polymorphic markers were scored for the presence or absence of bands.

Results and discussion

Callus induction

Most of explants of different genotypes produced a friable creamy-white callus at the cut end of the styles 7–10 days after the incubation (Figure 1) with the exception of mandarin explants which developed callus after 12 days. The variance analysis showed that the frequency of callus induction was significantly affected by media, species and genotype. Indeed, explants of the 4 citrus species grown in control medium (MS I) showed a lowest percentage of calluses (44.5% - 0%) as compared to explants cultured in BAP supplemented medium (MS II) (30% -100%) (Table 1). After a similar experiment [5] reported explants grown in control medium generally showed higher browning rate than explants cultured on the BAP

Explants excised from grapefruit growing in MS II showed high rate of browning 2 weeks after the culture initiation and 6 weeks after for mandarin explants. By contrast, no calluses were observed on MS I medium for both species.

Periodically, after 4-6 weeks from the culture initiation, callus from all genotypes were subcultured in the same culture conditions described above.

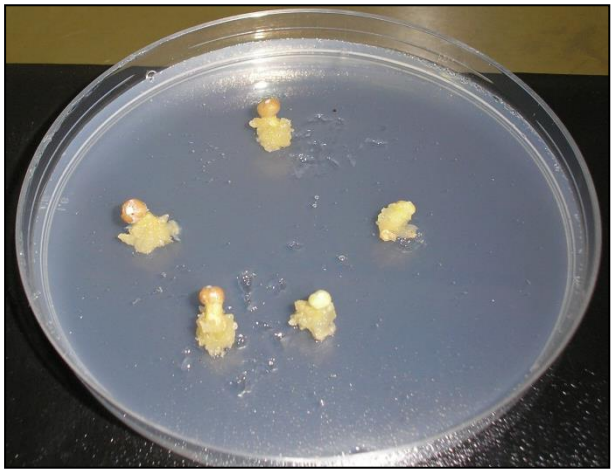

Figure 1. Callus formation from ‘Thomson’ navel orange

Table 1. Percentage of callus formation from different Citrus species.

Species	Genotype	Callus development / medium			
		MS I		MS II	
		%	Size	%	Size
C. sinensis (L.) Osbeck	Tarocco	-	+	100	++
	Washington navel	5	+	100	+++
	Alger navel	40	+	88.88	+++
	Orange de Blida	7,5	+	100	++
	Orange de Bey	-	+	100	++
	Double fine	-	+	100	++
	Metidja navel	44,5	+	100	+++
	Campbell valencia	-	+	100	++
	Thomson navel	-	+	100	+++
C. limon (L.) Burm	Lisbonne 16	40	++	100	+++
	Femminello	40	++	88.5	+++
	Dellys	40	++	100	+++
	Beni Abbés	24	++	100	+++
	Benni Haoua	13.68	++	72.5	+++
	Chlef	16.66	+	43.52	++
	Eureka maroc	10	++	60	+++
	Eureka 45	13.33	++	73.77	+++
	Secile	-	-	100	++
	Afrique du Nord	-	-	86.66	+++
	Lunario	-	-	30	++
	Bornéo	-	-	100	+++
C. reticulata Blanco	Blida	-	-	60	+
	Cleopatra	-	-	40	+
C. paradisi Macfad	Pink grapefruit	-	-	48	+

	Common grapefruit	-	-	43	+

Callus size: +++ (2-3cm); ++ (1-2cm); + (less than 1cm)

Somatic embryo development

Somatic embryos developed 25-90 days after culture initiation according to the genotype. Somatic embryos were green and easy to detach (Figure 2). Different embryo stages (globular, heart-shaped, torpedo and cotyledon stage) were observed as shown in Figure 3. The percentage of responsive styles varied from 3.30% to 50%. The variance analysis showed the embryogenic response of the explants was significantly influenced by media and genotypes (Table 2). Previous reports indicated that the addition of BAP to the medium greatly increased the embryogenic response of styles[9]. Indeed, in our experiment, explants cultured on MS II medium showed a greater potential response in terms of the percentage of embryos produced, while when explants were cultivated on MS I medium, not all genotypes responded in a positive way.

All tested lemon genotypes were successfully regenerated from stigma and style as previously reported [4]. Nevertheless, the highest somatic embryogenesis rate was obtained with the local varieties ‘Beni Haoua’ and ‘Chlef’, with 50% and 43.33% respectively in MS II, and 10% in MS I. The potential embryogenic rate of the other lemon varieties as ‘Afrique du nord’ (26.66%), ‘Eureka maroc’ and ‘Femminello’ (23.33%) was lower but anyway consistent and very promising results were also obtained with the Algerian genotypes ‘Beni Abés’ and ‘Dellys’ (16.66% and 6.66% respectively). In accordance with previous reports [10-11], the success of somatic embryos regeneration in lemon depends on the genotype, thus a great variability is shown within the same species.

[6] mentioned that not only lemon but also sweet oranges, particularly those belonging to the Navel group, showed a higher regeneration potential. Indeed, relatively to the cultured sweet orange genotypes (Table 1), embryos were obtained only with Thomson navel and with the local variety Metidja navel, both showing an homogeneous number of embryos with no significant differences in MS II (3.33%). Differently form the behaviour of other genotypes, Metidja navel doubled the number of embryos in MS I that were also forming more rapidly than those obtained in MS II (Table 2).

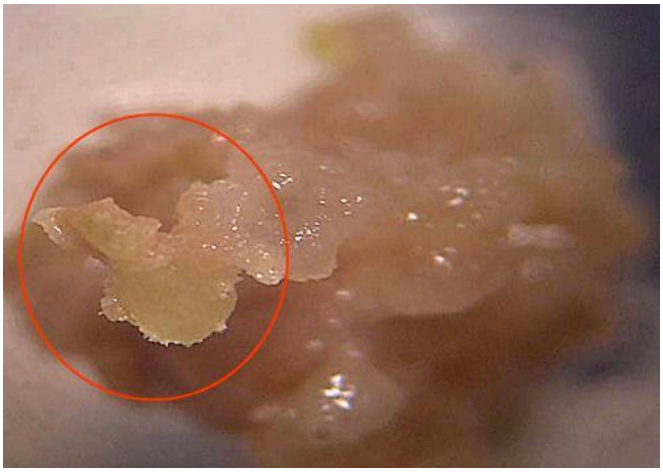

Figure 2. Embryos arising from *C. limon* (Benni haoua)

after 38 days of incubation

The attempts to induce somatic embryogenesis in all mandarin and grapefruit genotypes explants were unsuccessful. However,[6] reported that mandarin and grapefruit showed a lower percentage of embryo regeneration.

Table 2. Percentage of styles and stigmas producing somatic embryos from different citrus species

Species	**Genotype**	**SE formation (day)**		**Embryogenic explants (%)**	
		MS I	**MS II**	**MS I**	**MS II**
C. sinensis	Thomson navel	-	90	-	3.33
	Mitidja navel	50	90	6.66	3.33
C. limon	Lisbone 16	-	40	-	3.33
	Femminello	-	51	-	23.33
	Dellys	25	30	-	6.66
	Beni-Abés	69	69	3.33	16.66
	Beni-Haoua	38	38	10	50
	Chlef	32	39	10	43.33
	Eureka maroc	25	30	3.33	23.33
	Eureka 45	46	41	3.33	3.33
	Lunario	-	66	-	20
	Secile	-	64	-	3.33
	Afrique du nord		46		26.66
	Bornéo		90		3.33

Germination of somatic embryos

Embryos differentiated on the surface of the callus. Then, they were transferred to germinate in Petri dishes containing MS I medium . Even the rate of germinated embryos varied according to

species/genotypes; in fact, the frequency of the germinated embryos into plantlets was higher in lemon (75%) than in sweet orange (30%)**.** Similar results, 80% and 58% respectively, were reported by [12]. Generated plantlets were grown in tube for 2 months (Figure 4) before the acclimatization phase *in vivo*. When plantlets were 2-3 cm in length, they were grafted onto sour orange as described by [13].

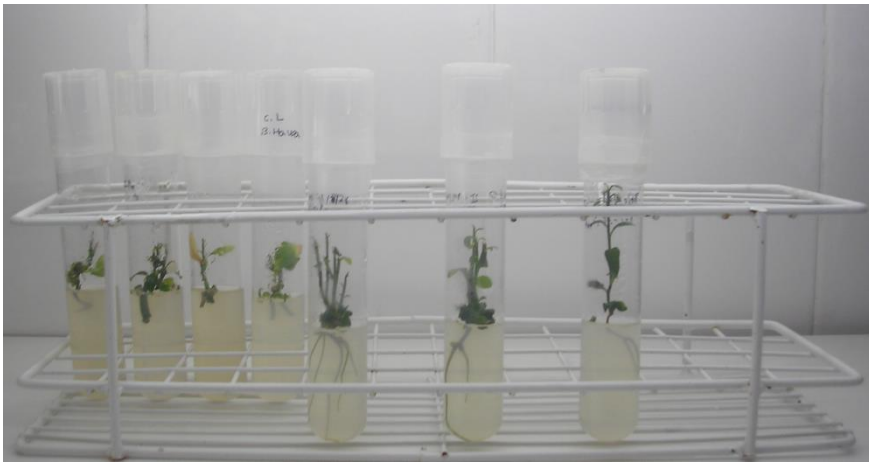

Figure 4. Plantlets from germinated embryos of *C. limon* (Beni-Haoua)

Genetic fidelity analysis of the regenerated plants

All regenerated plantlets appeared completely identical to the respective mother plants. Excepted The plants regenerated from metidja Navel genotypes showed genetic polymorphic fragment for 8 primers, while in W. Navel genotypes, the mother and micropropagated plants showed the same profile only for ISSR12-12.

Conclusion

Preliminary results of this investigation indicated that somatic embryogenesis using stigma and style culture has been successfully applied to regenerate different citrus genotypes of the main citrus species (sweet oranges and lemons) grown in Algeria. Since the tested genotypes were local or international varieties grown in the country for a long time, the successful results obtained by *in*

vitro SE regeneration represent the very first report. In accordance with previous works[10], regenerated genotypes belonged to lemon and sweet orange species, even if the cultured navels were expected to perform better. However, all lemon genotypes were regenerated but showing a different embryogenic rate. On the contrary, the callus formation in sweet oranges was mainly equal to 100%, higher than in lemon, but only 2 navels were able to produce somatic embryos at a very low rate. Nevertheless, native genotypes showed the best embryogenic potential achieved in the shortest period of time compared to the international ones. Further studies need to be carried out in order to investigate the embryogenic potential of other citrus genotypes of economical importance for the Algerian citriculture.

Citrus regenerated through stigma/style somatic embryogenesis proved to be true-to-type[14] and free from the main virus and virus-like diseases [6] Moreover, the successful and easy application of this technique under Algerian conditions showed that SE can be largely applied not only for citrus *in vitro* conservation but also for the production of healthy citrus plants to start up the citrus certification program in the country.

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