Ability of 'Flying Dragon' trifoliate orange to maintain high in vitro regenerative potential and polymorphism analysis by RAPD markers

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Ability of 'Flying Dragon' trifoliate orange to maintain high in vitro regenerative potential and polymorphism analysis by RAPD markers.

Abstract — Introduction. The 'Flying Dragon' trifoliate orange (FD) has been considered a truly dwarf rootstock, able to reduce, with most citrus scions, tree size by 75% of standard combination. The low availability of seeds, as well as the high production of zygotic seedlings made its micropropagation interesting. The results of 14 years of in vitro micropropagation of this rootstock are reported in this paper, in relation to genetic stability and regenerative capacity. **Materials and methods**. The explants were maintained in vitro on a modified MS medium supplemented with 6-benzylaminopurine and indole-3-butyric acid, through periodical cultures. Total DNA was extracted from leaves of 11 cultures grown in vitro and of a tree FD grown in the field as control. Random amplified polymorphic DNA analysis was performed using 60 decamers with arbitrary sequences. **Results**. In spite of the 14 years of micropropagation, the explants saved unaltered their morphological aspect and capacity of multiplication and rooting, but, in some cultures, RAPD analysis revealed the presence of polymorphism due to somaclonal variations. **Discussion**. According to the results obtained, in the first years of subculture, somaclonal variations did not occur. Micropropagation would be a reliable technique for germplasm preservation. Applied also in commercial laboratories for FD and other citrus cultivars, the micropropagation technique could be useful to propagate monoembryonic hybrids potentially interesting as rootstocks and rare *Citrus* species for ornamental uses. (© Elsevier, Paris)

***Citrus* / varieties / micropropagation / in vitro regeneration / somaclonal variations / polymorphism**

Capacité de l'oranger Flying Dragon à maintenir un fort potentiel de régénération en culture in vitro et analyse de son polymorphisme à l'aide de marqueurs RAPD.

Résumé — Introduction. L'oranger Flying Dragon (FD) est considéré comme un porte-greffe véritablement nanisant, capable de réduire de 75 % la taille de la plupart des variétés d'agrumes. Sa faible production de graines, associée à son aptitude à produire un fort taux de plantules zygotiques, rend sa micropropagation intéressante. Cet article permet d'analyser les résultats de 14 années de micropropagation in vitro de ce porte-greffe à la lumière de la stabilité génétique et de la capacité régénérative du matériel obtenu. **Matériel et méthodes**. Les explants de FD ont été maintenus en culture in vitro par cultures successives sur un milieu MS enrichi en 6-benzylaminopurine et en acide 3-indole-butyrique. Du DNA total a été extrait des feuilles de 11 cultures issues d'in vitro ; l'extrait témoin provenait d'un arbre du cultivar FD suivi en verger. Une analyse par RAPD a été effectuée sur le DNA à l'aide de 60 décamères à séquences arbitraires. **Résultats**. Malgré les 14 années de micropropagation, l'aspect des explants et leur capacité à se multiplier et à raciner sont restés intacts, mais, dans certaines cultures, les analyses par RAPD ont révélé du polymorphisme dû à des variations somaclonales. **Discussion**. D'après les résultats obtenus, les variations somaclonales ne se produisent pas pendant les premières années de culture. La micropropagation pourrait donc être une technique fiable pour la conservation du matériel génétique. Utilisée également en laboratoires commerciaux pour la multiplication de FD et d'autres cultivars d'agrumes, cette technique pourrait s'avérer utile pour la propagation d'hybrides monoembryonnés potentiellement intéressants et celle d'espèces rares d'agrumes exploitables en plante d'ornement. (© Elsevier, Paris)

***Citrus* / variété / micropropagation / régénération in vitro / variation somaclonale / polymorphisme**

1. introduction

The trifoliate orange 'Flying Dragon' (FD) [*Poncirus trifoliata* var. *monstrosa* (T. Ito) Swing.] is considered a genetically dwarfing species [1], able to reduce the size of most citrus cultivars grafted on it. Its effectiveness in high density plantings has been widely demonstrated [1–3]. Even if the FD is considered able to generate nucellar embryos, erect seedlings (zygotic), different from the typical zigzag vegetative habitus of the true-to-type trees, are often obtained. In some cases, this percentage can reach the 76% mark [4]. The lack of uniformity is a drawback to its diffusion. This stimulated the application of micropropagation technique to this rootstock which not only led to the uniformity of the material obtained but was also able to satisfy the demand of the market when consistent amounts of seed were not available. Furthermore, micropropagation allows to obtain great amounts of plants in little time in small areas, even in areas far from the cultivation sites, avoiding high expenses for maintaining numerous seed mother trees.

This paper refers to a work, started in 1983 at the *Istituto Sperimentale per l'Agrumicoltura* of Acireale, on the study of the in vitro multiplicative and rooting capacity of the FD explants and the verification of their genetic stability by RAPD analysis after 14 years of subcultures.

2. materials and methods

2.1. in vitro culture

The starting material used for micropropagation was excised in 1983 from a nucellar FD seedling, grown in the screenhouse. It was made up of nodal stem segment about 1–2 cm long, containing only one bud. The medium utilized was a modified MS medium [5] supplemented with 500 $mg \cdot L^{-1}$ malt extract, 25 $mg \cdot L^{-1}$ adenine, 50 $g \cdot L^{-1}$ sucrose and 8 $g \cdot L^{-1}$ agar. The pH of the medium was adjusted at 5.7 before autoclaving for 20 min at 120 °C.

Afterwards, shoots 1 cm long arising from the axillary buds of nodal stem segments were used for multiplication in the already mentioned MS medium supplemented with 1 $mg \cdot L^{-1}$ 6-benzylaminopurine (BA) and 0.5 $mg \cdot L^{-1}$ 3-indolbutyric acid (IBA).

Every 5 or 6 weeks, each 'multiple shoots' formation was divided into single shoots that were multiplied through subcultures in the same fresh medium. Each subculture, at transferring time, was registered by numbering progressively and indicating the origin, the substrate, the culture date and the total number of explanted shoots derived from each single 'multiple shoots' formation.

To induce rooting, single shoots 1.5 cm long were placed onto the modified MS medium supplemented with 1 $mg \cdot L^{-1}$ naphtaleneacetic acid (NAA).

The cultures were incubated in a growth-chamber at 27 ± 1 °C with 16 h per d low intensity light (1 000 lx) from 40 w·77 L Fluora fluorescent lamps.

The 5 cm high rooted-shoots were transplanted into peat pots filled with sterilized soil and grown in a hotbed frame under 100% humidity and temperature 25–27 °C, for about 1 month and then transplanted in 21 cm diameter pots and cultivated in greenhouses.

2.2. DNA amplification

For DNA analysis, samples were selected on the basis of their diversification period, taking into account the most representative available material.

A RAPD analysis was performed on genomic DNA extracted from each 'multiple shoots' formation derived from cultures growing in vitro and from mother tree of FD growing in the field as control. The explants, made up of single shoots without leaves, which instead were used for DNA amplification, were maintained in vitro.

Total DNA was extracted from 20–40 mg of shoots, according to Yu and Pauls [6].

The polymerase chain reaction (PCR) was performed in a volume of 25 µL containing 2.5 µL buffer 1X, 3 mM $MgCl_2$, 10 mM of dNTPs, 5 pM primer 1.0 unit of Taq polymerase (Perkin Elmer) and 25 ng of genomic DNA. Reactions were performed in a thermal-cycler Perkin Elmer Gene Amp PCR System 9600, with a step of denaturation at 94 °C for 5 min, 45 cycles, each of them performed at 94 °C for 15 sec, 36 °C for 15 sec and 72 °C for 80 sec followed by a step of elongation at 72 °C for 10 min. A total of 60 random decamer oligonucleotide primers (H - K - P from Operon Technologies Inc., Alameda, USA) were used to amplify genomic DNA. Amplification products were separated on 1.5% agarose gel in TAE buffer and visualised with ethidium bromide staining.

3. results

3.1. in vitro culture

Three or four shoots were produced from a primordial nodal stem cultured on MS medium without cytokinin and auxin [7]. After 4 weeks, they were excised and placed on the already mentioned MS medium supplemented with BA and IBA. In this first phase, each of them produced an average of five new shoots after 8 weeks [8].

From then, the explants produced numerous and vigorous 'multiple shoots' formations with well developed internodes and leaves. The shoots were separated individually and used for multiplication or for rooting when longer than 1.5 cm. At this point a single shoot explant, subcultured on the same fresh propagation medium, yielded an average of 19 shoots every 35–40 d. Roots developed easily when the shoots were placed on MS medium added with 1 $mg \cdot L^{-1}$ of NAA [9].

After 115 subcultures, the multiplication ability of the explants remains very high.

3.2. DNA amplification

The data saving procedure allowed to ascertain the explants origin and the period when each of them diversified (*figure 1*). In the frame of 11 cultures examined, number 5238 diversified in 1987, the others in more recent time. For such reason, presumably, this culture accumulated a greater number of mutations. Thus it was chosen together with no. 5409 and with an FD grown in field, for a preliminary RAPD analysis, based on a screening made with 60 primers.

Among the primers examined, K09, K16, K17 and H17 highlighted polymorphic fragments. RAPD analysis was performed on the other nine cultures as well, using the same primers, but only the amplification with K09, repeated three times, expressed satisfying reproducible or sufficient intensity polymorphisms.

Thus RAPD analysis performed with K09 demontrated that in vitro culture is able to induce somaclonal variations (*figure 2a*). In fact, the no. 5409 (lane 2; *figure 2a, b*) presents a band (988 bp) absent in the control. The subculture 5271 (lane 3; *figure 2a*), having in 1993 the same common origin as no. 5409, shows the same polymorphism and, in addition, a band of 928 bp, absent in 5409 and pre-

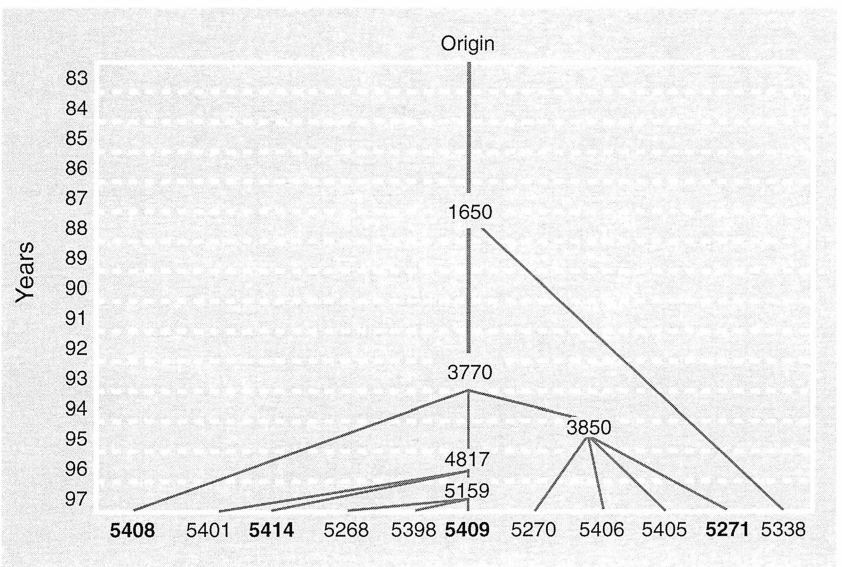

Figure 1.
Origin of citrus subcultures used for the DNA amplification in order to study the polymorphism of plants micropropagated in vitro.
The bold numbers indicate where presence of polymorphism has been revealed.

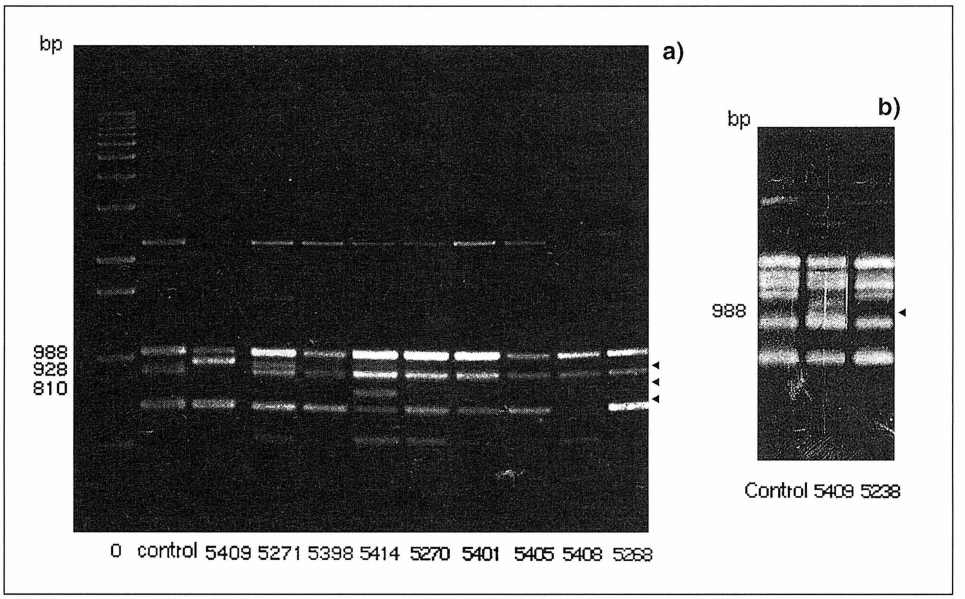

Figure 2a, b.
Citrus subculture DNA amplification by OPK09 primer.
0 = molecular marker 1 kb (from Gibco BRL);
control: Flying Dragon tree growing in the field.

sent in the control and in the other subculture; the culture no. 5414 (lane 5; *figure 2a*) presents a unique polymorphism of 810 bp; the culture no. 5238, which differentiated from the other subcultures in 1987, presents the same polymorphism as the control and the six others (no. 5401, 5268, 5398, 5270, 5406 and 5405) (*figure 2a, b*).

4. discussion and conclusion

After 14 years of micropropagation, the single shoot explants maintain their regenerating and rooting ability; plants obtained with this method do not show any apparent variation in the vegetative habitus.

The absence of polymorphism in culture no. 5238 deriving from no. 1650 led us to think that during the first 5 years of propagation no mutations sorted. Polymorphic fragments were found only in cultures no. 5408, 5409, 5414 and 5271, diversified after 1993. This allowed to establish that, even if they originated from the same strains (no. 3770), the cultures accumulated mutations of different nature, but in more recent time.

At present, we cannot affirm if these somaclonal variations are solid or chimeric, heritable or epigenetic, and if the polymorphisms are located in areas of genome coding for physiological characteristics of relevance or for repetitive sequences not coding.

From our experience, we can conclude that, in order to avoid the appearance of somaclonal mutations, a 5-year period of micropropagation could be considered reliable for germplasm conservation. Thus, the micropropagation technique can be used as an alternative method of germplasm conservation paying attention to limiting the number of subcultures and to frequently renewing the primary source.

The micropropagation technique has also been applied in commercial laboratories for 'Flying Dragon' and 'BA 300' citrange [7], an interesting partial dwarfing rootstock isolated in seedbed of Troyer citrange, after 19 years still without flowering. It could also be useful to propagate monoembryonic hybrids potentially interesting as candidate rootstocks and rare *Citrus* species, also for ornamental uses.

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Capacidad del naranjo Flying Dragon a mantener un fuerte potencial de regeneración en cultivo in vitro y análisis de su polimorfis mediante marcadores RAPD.

Resumen — Introducción. Se considera al naranjo Flying Dragon (FD) como un porta injerto con verdadera capacidad a producir enanos, capaz de reducir de un 75 % el tamaño de la mayoría de las variedades de agrios. Su baja producción de semillas, asociada con su aptitud a producir una importante tasa de plántulas zigóticas, hace su micro propagación interesante. Este artículo permite analizar los resultados de 14 años de micropropagación in vitro de este porta injerto a la luz de la estabilidad genética y de la capacidad regenerativa del material logrado. **Material y métodos**. Se mantuvieron las explantes de FD en cultivo in vitro mediante cultivos sucesivos en un medio MS enriquecido en 6-benzilaminopurina y en ácido 3-índole-butírico. Se ha extraído DNA total de hojas de 11 cultivos procedentes de in vitro; el extracto testigo provenía de un árbol del cultivar FD estudiado en huerto. Se realizó un análisis por RAPD en el DNA mediante 60 decameros de secuencias arbitrarias. **Resultados**. A pesar de los 14 años de micropropagación, el aspecto de las explantes y su capacidad a multiplicarse y en enraizarse se quedaron intacta, pero, en ciertos cultivos, los análisis por RAPD revelaron polimorfismo debido a variaciones somaclonales. **Discusión**. Acorde a los resultados logrados, las variaciones somaclonales no ocurren durante los primeros años de cultivo. La micropropagación podría por lo tanto ser una técnica fiable para la conservación del material genético. Utilizada también en laboratorios comerciales para la multiplicación de FD y demás cultivares de agrios, esta técnica podría revelarse útil para la propagación de híbridos monoembrionados potencialmente interesantes y la de especies raras de agrios explotables en planta de adorno. (© Elsevier, Paris)

***Citrus* / variedades / micropropagación / regeneración in vitro / variación somaclonal / polimorfismo**