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Tissue culture of European accessions of persimmon: Callusing and proliferation

E. Giordani*, A. Garcés, R. Perria* and E. Bellini***
*Dipartimento di Ortoflorofrutticoltura, Università degli Studi di Firenze, Polo Scientifico, Viale delle Idee 30, 50019 Sesto Fiorentino (FI), Italy
E-mail: edgardo.giordani@unifi.it
**Institut de Recerca i Tecnologia Agroalimentària, Apartado 415, 43280 Reus, Tarragona, Spain

SUMMARY – *In vitro* establishment and callus formation are two important steps in tissue culture for regenerative purposes in persimmon. The suitability to tissue culture and the callusing activity was assessed on European varieties (namely 'Cioccolatino', 'Mercatelli', 'Moro', 'Rispoli' within the Italian germplasm, and 'Betera 1', 'Enguera 1', 'La Selva', 'Reus 15', 'Rojo Brillante', 'Tomatero', 'Xato del Bon Repos' and 'Picudo', among the Spanish varieties) in comparison to Japanese cultivars ('Jiro' and 'Tone Wase') and to 'Kaki Tipo'. Explants from dormant buds were cultured in MS (1/2 N) basal medium, with 20 g/l of sucrose, solidified with ICN - Gel Gro (Gellum gum), added with Zeatin (10 μM), Indole-3-Acetic Acid (1 μM). After 60 days of culture in the dark at 23°C (±0.5) calluses were measured and weighted. The best results were obtained on 'Tone Wase', 'Tomatero', 'Reus 15', 'Rojo Brillante', 'Xato del Bon Repos', 'La Selva' and 'Picudo', with very active white calluses. The suitability to micropropagation of 'Rojo Brillante', a very important PCA cultivar in the Spanish persimmon industry, was assessed.

Key words: *Diospyros kaki*, tissue culture, callusing, micropropagation.

RESUME – ***"****Culture de tissus chez des accessions européennes de plaqueminier : Formation de callus et prolifération". L'établissement* in vitro *et la formation de callus sont deux étapes importantes de la culture de tissus à des fins de régénération chez le plaqueminier. L'adaptation à la culture de tissus et à la formation de callus a été évaluée chez des variétés européennes (à savoir 'Cioccolatino', 'Mercatelli', 'Moro', 'Rispoli' pour le germoplasme italien, et 'Betera 1', 'Enguera 1', 'La Selva', 'Reus 15', 'Rojo Brillante', 'Tomatero', 'Xato del Bon Repos' et 'Picudo', parmi les variétés espagnoles) en les comparant aux cultivars japonais ('Jiro' et 'Tone Wase') et à 'Kaki Tipo'. Des explants de bourgeons dormants ont été cultivés en milieu basal MS (1/2 N), avec 20 g/l de sucrose, solidifié à l'ICN - Gel Gro (gomme Gellum), additionné de zéatine (10 μM), Acide Indole-3-Acétique (1 μM). Après 60 jours de culture sous obscurité à 23°C (±0.5) les callus ont été mesurés et pesés. Les meilleurs résultats ont été obtenus sur 'Tone Wase', 'Tomatero', 'Reus 15', 'Rojo Brillante', 'Xato del Bon Repos', 'La Selva' et 'Picudo', avec des callus blancs très actifs. On a évalué l'adaptation à la micropropagation de 'Rojo Brillante', un cultivar PCA très important pour l'industrie du plaqueminier en Espagne.*

Mots-clés : Diospyros kaki*, culture de tissus, formation de callus, micropropagation.*

Introduction

The first experiments on the culture of *Diospyros kaki* tissues began in Japan in the 1970's, assuming importance for genetic improvement through modern biotechnological techniques. The use of classic genetic improvement methods through crossing in *D. kaki* is considerably blocked by the high ploidy and complex sex expression typical of the species, which render selection difficult and self-fertilization very often impossible. In addition, the development of a valid micropropagation technique would favour the spread of clonal rootstocks, given that currently all rootstocks employed are obtained from seed with poorly known origin and characteristics. Further, it would be interesting to be able to utilize *in-vitro* self-rooted cultivars, overcoming the big difficulty of *in vivo* rooting of the species and problems related to a lack of graft affinity.

In vitro establishment and callus formation are two important steps in tissue culture for regenerative purposes in fruit species. Since 1976 efforts have been made in Japan with regard to persimmon tissue culture and to callusing and regeneration (Niimi and Yamamoto, 1987; Tao and Sugiura, 1992). In Italy, the Department of Horticulture of the University of Florence has studied the

definition of protocols for the *in vitro* regeneration from callus of persimmon for different cultivars. In previous works, regeneration was obtained for 'Jiro', 'Cal-Fuyu', 'Maekawa Jiro', 'Hana Fuyu', 'Tenjin Gosho' and 'Kaki Tipo'. A larger set of cultivars were used in the present work, placing greater importance on European cultivar trials (Bellini and Giordani, 1994; Bellini *et al.*, 1996; Innocenti, 1996).

Interesting results have been obtained on *in vitro* proliferation and micropropagation protocols were defined for some cultivars (Cooper and Cohen, 1984; Murayama *et al.*, 1989; Fukui *et al.*, 1990; Sarathchandra and Burch, 1991; Tao, 1992); in the 1990's studies were begun at the Department of Horticulture of the University of Florence evaluating the possibility of obtaining *in vitro* proliferation of shoots of the cultivars 'Jiro' and 'Kaki Tipo', the most important cultivar in Italy (Bellini and Giordani, 1997; Folli, 1998).

No analogous studies have been carried out on the cultivar 'Rojo Brillante', an important Spanish PCA (pollination constant astringent) cultivar. In recent years, the Spanish production of 'Rojo Brillante' has reached considerable development, largely covering the request for persimmons on the European market (Climent and Llacer, 2001). Thus, there is the need to test this cultivar for its attitude for *in vitro* growth, through proliferation of shoot apexes.

In vitro establishment and callus formation

Taking into account previous experiences on callus formation from bud explants carried out on 'Jiro', the callusing activity was assessed on presumed European varieties (namely 'Brazzale', 'Cioccolatino', 'Kaki Tipo', 'Mercatelli', 'Moro', 'Rispoli' within the Italian germplasm, and 'Betera 1', 'Costanti 13', 'Enguera 1', 'La Selva', 'Reus 15', 'Rojo Brillante', 'Tomatero', 'Xato del Bon Repos' and 'Picudo', among the Spanish varieties) in comparison to non European cultivars of *D. kaki* L.f. ('Jiro', 'Fuyu', 'Giant Fuyu', 'Tone Wase', 'Triumph') and of 'Shinano Kogaki', an accession of *D. lotus* L.

The starting plant material was composed of axillary buds removed during January and February from the persimmon collection at the Montepaldi farm belonging to the School of Agriculture of the University of Florence. The grafts were kept in a refrigerated cell at 4°C until their use.

Explants from dormant buds were sterilized in a 3.5% HClO solution and 0.1% Tween 20, under stirring for 7 min. After having removed the scales, apexes were cultured in MS (1/2 N) basal medium, with 20 g/l of sucrose, solidified with ICN - Gel Gro (Gellum gum), added with Zeatin (10 μM) and Indole-3-Acetic Acid (1 μM) in Petri dishes.

The capsules were kept in the dark at 23°C (+0.5) for 30 days, then their weight and projection of callus surface were measured for the first time; the second measurement was taken 60 days after culture. Calluses larger than 1 cm^2 of surface projection were divided and transferred to the same medium and divisions continued as the calluses reached this size, afterwards they are transferred to the regeneration medium under light.

After 30 and 60 days of culture in the dark at 23°C (±0.5) calluses were measured and weighed. All cultivars, apart from 'Costanti 13' (*D. kaki*) and 'Shinano Kogaki' (*D. lotus*) formed calluses, which often appeared dark, small and not very active in their growth. Data relative to calluses of measurable dimensions are reported ('Tone Wase', 'Jiro', 'Tomatero', 'Picudo', 'Rispoli', 'La Selva', 'Enguera 1', 'Moro', 'Cioccolatino', 'Reus 15', 'Xato del Bon Repos', 'Betera 1', 'Rojo Brillante', 'Kaki Tipo', 'Mercatelli') (Fig. 1). The calluses obtained from buds of other cultivars reached irrelevant size and stopped growing immediately, and necrotized.

The best results were obtained on 'Tone Wase', 'Tomatero' and 'Picudo', with very active white calluses larger than those of 'Jiro'; despite the characteristics of the calluses obtained from these three cultivars, they appear very different among them. Calluses of 'Tone Wase', like those of 'Jiro', are from white to light yellow in colour and grow very quickly; calluses of 'Tomatero' are generally darker in colour in the centre and lighter on the outside; 'Picudo' calluses are black from the beginning of their formation. Generally, callus characteristics vary from cultivar to cultivar but remain constant within the same cultivar.

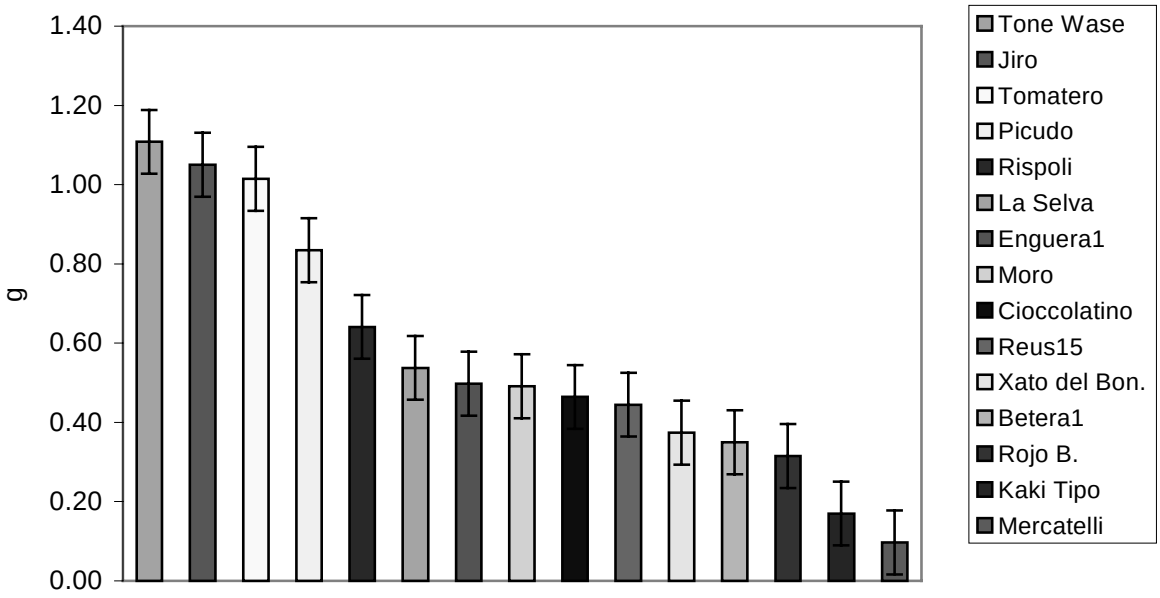

Fig. 1. Mean weight reached by calluses after 60 days of culture.

Proliferation of 'Rojo Brillante'

Evaluation of the attitude of 'Rojo Brillante' for *in vitro* proliferation was carried out by comparison with the cultivar 'Jiro', employing protocols already developed for persimmon.

The initial plant material was composed of axillary buds removed during the vegetative dormancy phase of persimmons grown at the Montepaldi farm of the School of Agriculture of the University of Florence. Grafts were removed at the beginning of January and kept in a refrigerated cell at 4°C until the time of their use. Buds, removed with a scalpel, were disinfected with a 3.5% NaClo solution with the addition of 0.1% Tween 20 under stirring for 7 min. From the sterilized buds the scales were removed and then placed in culture in Petri dishes on a medium of MS 1/2N, with 20 g/l sucrose, solidified with ICN - Gel Gro (Gellum gum) added with 10 µM zeatin under the following environmental conditions: temperature 25 ± 1°C, photoperiod 16 h fluorescent light (40 µmol/m^2/s).

The shoots derived from the buds were transferred into from Petri dishes to glass vessels containing 50 ml of the same culture medium; the medium was renewed every 30 to 40 days.

The methodology of sterilization utilized gave optimal results in that no contamination was noted and all the buds remained vital.

After two months, a few shoots (2.3 for 'Jiro' and 2.4 for 'Rojo Brillante') originated from all the buds in culture; after six months the shoots/bud average increased for 'Jiro' (4.6) and decreased for 'Rojo Brillante' (2.3), with an average shoot length of 11.40 mm for 'Jiro' and 20.90 mm for 'Rojo Brillante' (Fig. 2). In both cultivars, the number of shoots/bud decreased in the first subculture and then underwent a rapid increase until the fifth subculture and thereafter (Fig. 3). This behaviour is probably due to the slow adaptation of the plantlets to the *in vitro* conditions.

Notable differences were not found between the conditions of the 'Jiro' and 'Rojo Brillante' shoots with regard to colour, aspect and leaf dimensions; however it can be said that the 'Rojo Brillante' shoots lengthened more quickly and can be introduced earlier into the rooting phase.

The protocol developed for the Japanese cultivar 'Jiro' is easily adaptable to the Spanish cultivar 'Rojo Brillante'. Even if at present, the proliferation coefficient remains very low and the costs for production very high, valid prospects exist for the micropropagation of this cultivar.

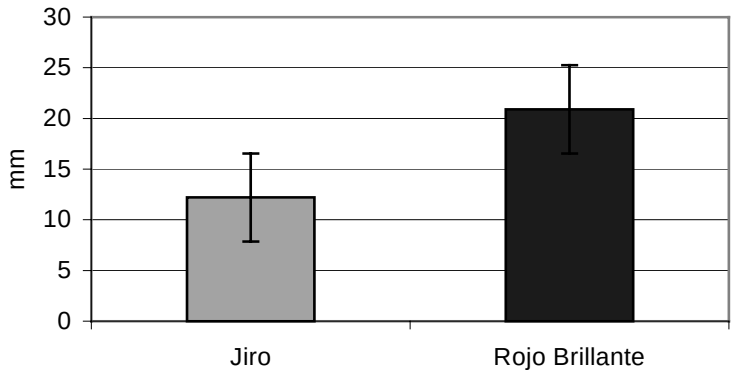

Fig. 2. Mean length of shoots after 3 sub-cultures.

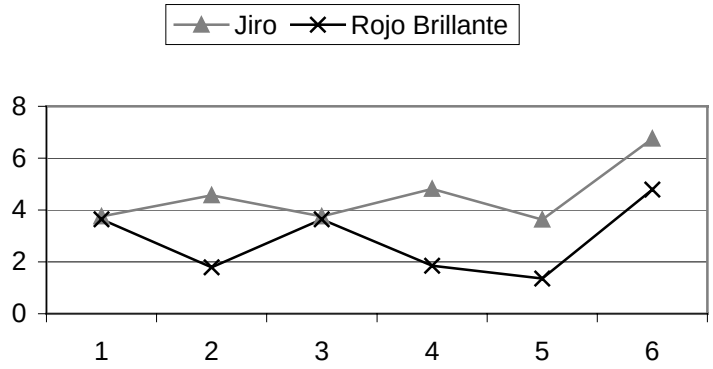

Fig. 3. Number of shoots/bud for increasing sub-cultures.

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