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In vitro Plant Regeneration from Callus of *Citrus x monstruosa* (Pompia), an Endemic Citrus of Sardinia

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A regeneration protocol was developed from callus obtained from various explants taken from *in vitro* cultured seedlings (root, leaf and stem internodes) of *Citrus x monstruosa*. The best treatment in terms of response frequency and mean number of shoots for explants was 35.0 µM BA with 5.5 µM NAA. The best shoot regeneration was obtained with internodal stem segments cut longitudinally with the cut surface in contact with the culture medium and pre-treatment of 21 days of these explants in darkness. The best rooting of explants was obtained on half-strength MS basal medium supplemented with either NAA or IBA at 5.4 µM and 2.5 µM, respectively. Hardening of *Citrus* x *monstruosa* was accomplished in 40 days, with 95% survival rate.

Keywords: *Citrus x monstruosa*, Pompia, callus culture, *in vitro* regeneration, *in vitro* culture, organogenesis.

Citrus x monstruosa "Pompia" (Rutaceae) is a widespread endemic *Citrus* limited to and sporadic in the area of Baronia (eastern Sardinia) in the towns of Siniscola, Posada, Torpè, and Orosei, and in Triei, Ogliastra. The plant's ancient origin is unknown and is of uncertain taxonomic classification [1]. Cultivation is spreading because of the use of the fruits in pastry.

"Pompia" is a tree producing fruits with a low juice content, which has a low level of sugars, but high acidity [2]. The plant was characterized for the first time from a biochemical and biomolecular point of view by Mignani *et al*. [3], making it possible to determine the degree of similarity between "Pompia" and five species of *Citrus*. In particular, it was shown that the lemon and citron genotypes are closest to "Pompia". The information that emerged from this work also allowed the assumption to be made of the same clonal origin of the individuals studied from different places in Baronia.

In order to protect this important endemic citrus for the maintenance of the biodiversity in Sardinia, we have developed an efficient *in vitro* plant regeneration system.

In vitro Citrus plant regeneration via organogenesis has been described from several tissues, including roots [4], leaf sections [5-7], stem internodes [8,9], and *in vitro* cultured seedlings [10]. In this study, we have determined the optimal conditions for *in vitro* plant regeneration via organogenesis of *Citrus x monstruosa* "Pompia" using explants taken from *in vitro* cultured seedlings.

Citrus x *monstruosa* seeds germinated after 25-30 days (see Experimental), with a germination percentage of nearly 15%. The cultures of leaf and root explants from *in vitro* cultured seedlings did not produce adventitious shoots after two months on the tested media and under all conditions tested. The culture of leaf explants (Table 1) produced non-regenerating green and compact callus (**ngcc**) in condition A (16 h. photoperiod), and non-regenerating brown friable callus (**nbfc**) in condition B (pre-incubation for 21 days in darkness) when both BA and NAA were present, at all concentrations tested. In the presence of 33.1 µM BA alone, explants of leaves did not react under A conditions, but underwent necrosis (**nec**) after 20 days. Exposure of leaf explant callus obtained after pre-incubation for 21 days in darkness with a 16 h photoperiod (condition B) did not induce production of adventitious shoots, but necrosis after 10 days of exposure.

The presence in the medium of TDZ with either auxin (NAA) or with auxin (NAA) and cytokinin (BA) did not induce adventitious shoot production in leaf explants (conditions A and B), but non-regenerating callus (**nrc**).

Table 1: Effect of various combinations and concentrations of growth regulators on explants utilized: leaf, stem and root. (A) = 16 h. photoperiod; (B) = pre-incubation for 21 days in darkness.

Growth regulators (µM)			(A)			(B)		
			Leaf	Stem	Root	Leaf	Stem	Root
BA	TDZ	NAA						
10.2	-	5.5	ngcc	rc	nec	nbfc	rc	nec
21.2	-	5.5	ngcc	rc	nec	nbfc	rc	nec
35.0	-	5.4	ngcc	rc	nec	nbfc	rc	nec
33.1	-	2.5	ngcc	rc	nec	nbfc	rc	nec
42.5	-	2.5	ngcc	rc	nec	nbfc	rc	nec
33.1	-	-	nec	nrc	nec	nec	nrc	nec
-	0.50	5.5	nrc	rc	nec	nrc	rc	nec
-	0.90	5.5	nrc	rc	nec	nrc	rc	nec
10.2	0.25	5.5	nrc	rc	nec	nrc	rc	nec

ngcc-non-regenerating green and compact callus; nbfc-non-regenerating, brown, friable callus; nec-necrosis; nrc-non-regenerating callus; rc-regenerating callus

Table 2: Effect of various combinations and concentrations of growth regulators on adventitious shoot proliferation from internodal stem pieces of *Citrus* x *monstruosa*.
Data pooled from three experiments. (A) = 16 h. photoperiod; (B) = pre-incubation for 21 days in darkness.

Growth regulators (µM)			Adventitious shoots from explant* (no.±SE) (A)		(B)	
BA	TDZ	NAA	Vert.	Horiz.	Vert.	Horiz.
10.2	-	5.5	2.61±0.18	4.12±0.37	4.26±0.35	11.33±0.95
21.2	-	5.5	2.11±0.16	3.75±0.24	4.51±0.34	12.15±0.97
35.0	-	5.5	3.53±0.24	7.34±0.66	6.52±0.55	15.63±1.18
33.1	-	2.5	2.15±0.18	3.52±0.25	4.57±0.37	10.27±0.96
42.5	-	2.5	2.52±0.15	3.64±0.26	3.25±0.24	9.35±0.84
33.1	-	-	-	-	-	-
-	0.50	5.5	2.33±0.16	4.18±0.37	4.11±0.37	11.25±0.97
-	0.90	5.5	2.71±0.19	3.62±0,26	3.66±0.26	10.41±0.98
10.2	0.25	5.5	2.12±0.18	3.87±0.28	3.50±0.24	6.65±0.54

*Mean represents three replicates for each treatment, followed by standard deviation.
Statistical analysis was performed using the non parametric Kruskal-Wallis one-way analysis of variance.
The significance level was fixed at $\alpha = 0.05$.

Table 3: Effect of auxins in different concentrations on rooting of shoots derived from callus.

Auxins	Root number*	Root length* (cm)
Hormone free	-	-
NAA 2.5 µM	5.81±0.44	1.26±0.11
NAA 5.4 µM	8.34±0.76	1.85±0.16
IBA 1.0 µM	3.67±0.24	1.10±0.09
IBA 2.5 µM	7.52±0.68	2.27±0.19
IAA 1.5 µM	1.54±0.15	0.68±0.05
IAA 3.2 µM	1.71±0.16	0.72±0.06

*Mean represents three replicates for each treatment, followed by standard deviation.
Statistical analysis was performed using the non parametric Kruskal-Wallis one-way analysis of variance.
The significance level was fixed at $\alpha = 0.05$.

Under all conditions tested, root explants showed necrosis after 15 days of culture. Internodal stem explants inoculated vertically produced regenerating callus (**rc**) in media with auxin and cytokinin, and non-regenerating callus with cytokinin alone (**nrc**).

Considering that none of the cultures of leaf and root explants produced regenerating callus, we utilized internodal stem pieces for shoot induction and investigated the influence of the explant position and of light/ dark on regeneration. Table 2 reports shoot proliferation from vertically and horizontally incubated stem explants (see experimental) under both A and B culture conditions.

For the internodal stem segments inoculated vertically, with the apical end protruding from the culture, the number of shoots from explants originating from the apical zone alone ranged from 2.11±0.16 to 3.53±0.24 and 3.25±0.24 to 6.52±0.55 in culture conditions A and B, respectively (Table 2). The best response was obtained with internodal stem segments

Figure 1: Regenerated shoots from horizontally cultured internodal explant without pre-incubation of 21 days in the dark (condition A).

Figure 2: Regenerated shoots from horizontally cultured internodal explant with pre-incubation of 21 days in the dark (condition B).

cut longitudinally with the cut surface horizontally in contact with the culture medium; in these conditions the internodal explants produced green and compact callus on their entire surface. This procedure significantly increased the yield of regenerated adventitious shoots with production of shoots from each explant ranging from 3.52±0.25 to 7.34±0.66 (condition A) (Figure 1) and from 6.65±0.54 to15.63±1.18 (condition B) (Figure 2).

Pre-incubation in the dark for three weeks induced a significant increase in the production of adventitious shoots from the internodal explant used. (Table 2). This treatment had no effect on any other explant employed in any of the experimental conditions.

In accord with previous research [8], this experiment confirmed that increasing the area of the wounded surface also increases the production of adventitious shoots.

The best treatment in terms of the response frequency and mean number of shoots for explants was 35.0 µM BA with 5.5 µM NAA. In accord with previously published data [8], the adventitious shoots generated in the presence of TDZ exhibited abnormal leaf morphology.

The best rooting of explants in terms of root numbers was obtained with half-strength MS basal medium supplemented with either NAA or IBA at 5.4 µM and 2.5 µM, respectively (Table 3).

The roots appeared 5-6 weeks after transfer, and their length ranged between 0.68±0.05 to 2.27±0.19 cm. Roots formed on 80% of the transplanted shoots. No roots were formed in media without growth regulators and activated charcoal was necessary for root development. Rooted shoots were transplanted into plastic pots containing a mixture of vermiculite: peat moss (1:1). Hardening of *Citrus* x *monstruosa* was accomplished in 40 days, with 95% survival rate.

Our results suggest that regeneration from callus for eventual conservation of *Citrus* x *monstruosa* is possible through *in vitro* culture of internodal explants.

Experimental

Plant material: *In vitro* germinated seedlings of “Pompia” were used as the tissue source. Seeds for experiments were kindly supplied by Dott. P. Cara from Siniscola (Sardinia).The sterilisation process of seeds was conducted in 3 steps: washing for 20 min. in distilled water plus a commercial solution of a detergent (Amuchina); immersion in 50% ethanol for 1 min; and immersion in 0.1% $HgCl_2$ for 10 min under vacuum, followed by 5 rinses in sterile distilled water, and drying on sterile filter paper [11]. The germination of seeds occurred in 500 mL glass jars containing 100 mL culture medium modified according to Gavazzi *et al*. [12] plus 0.8% agar, pH 5.8. Germination took place in 3 months at 27 ± 2°C in a growth chamber with a photoperiod of 16 h. under fluorescent tubes at a light intensity of 65 µmol m^{-2} s^{-1} [13].

In vitro culture: The basal culture medium for regeneration was the inorganic salts of Murashige and Skoog [14] with the vitamins of GB5 [15] in accordance with Pérez-Molphe-Balch and Ochoa-Alejo [8] supplemented with 9 combinations and concentrations of growth regulators, 5% sucrose, 8 g/L^{-1}agar (Sigma); the pH was adjusted before autoclaving to 5.8. Growth regulators and sucrose were added before autoclaving.

Aliquots of 30 mL of the media were dispensed into 90 mm diameter Petri dishes. The effects of various combinations and concentrations of growth regulators on adventitious shoot formation from leaf, stem, and root explants of *Citrus x monstruosa* seedlings (obtained *in vitro*) with 4 to 6 internodes were tested.

The basal medium was supplemented with several combinations of BA (6-benzylaminopurine), TDZ (Thidiazuron) and NAA (1-Naphthaleneacetic acid) (Table 1).

For callus induction, the leaf segments were placed with their adaxial side in contact with the surface of the

regeneration medium; roots were cut into 10 mm segments and placed horizontally on the medium and 10 mm internodal stem pieces were inoculated vertically in the medium with the apical end of the segment protruding by 6 to 7 mm. For shoot regeneration from callus, a second condition was used for stems: the internodal stem pieces were cut longitudinally and the cut surface placed horizontally in contact with the culture medium [8].

Cultures were incubated at 25 ± 2°C with a 16 h. photoperiod under fluorescent lights with a photon flux of approximately 52 µmol m^{-2} s^{-1} : condition A; or at 25 ± 2°C for 21 days in darkness, followed by 50 days of incubation with a 16 h photoperiod, as above: condition B. The number of adventitious shoots obtained from calluses was recorded periodically. One hundred explants of each kind were used for treatment ant the entire experiment was carried out 3 times. The statistical data were evaluated for the regenerative ability of various groups of calluses. For root induction, shoots taller than 2.5 cm were transferred to half-strength MS medium with or without 200 mg L^{-1} activated charcoal and without growth regulators or with 2.5-5.4 µM NAA, 1.0-2.5 µM IBA or 1.5-3.2 µM IAA . Statistical analysis of the results was performed using the non-parametric Kruskal-Wallis one-way analysis of variance. To determine which pairs of samples tended to differ, a standard procedure of multiple comparison for the Kruskal-Wallis test was used. The significance level was fixed at $\alpha = 0.05$.

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