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Direct Organogenesis from Cotyledons in Cultivars of *Citrus clementina* Hort. Ex Tan

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ABSTRACT

An efficient protocol to induce shoot buds regeneration in Citrus clementina cultivars ("*Monreal*", "*SRA* 63" *and* "*SRA* 64") *by direct organogenesis has been developed using cotyledons as explants. Cotyledons transversely cut in three segments and entire ones were cultured on Murashige and Skoog* (1962) *solidified medium containing vitamins*, 500 $mg \cdot l^{-1}$ *malt extract*, 50 $g \cdot l^{-1}$ *sucrose and supplemented with three different concentrations of BAP* (8.8, 13.2 *and* 17.6 *μM*). *In all three cultivars the entire cotyledons showed more shoot morphogenic potential than transversely cut ones and after* 60 *incubation days the optimum BAP concentration was* 17.6 *μM in* "*Monreal*" (50% ± 2.89% *of frequency regeneration*) *and* 13.2 *μM in* "*SRA* 63" (33.33% ± 3.33%) *and* "*SRA* 64" (25.93% ± 1.85%). *In absence of BAP No morphogenesis occurred*, *demonstrating the absolute requirement of this hormone for shoots induction. The young shoots showed a regular growth in the culture tubes containing the basal medium without hormones*, *and the rooted plantlets survived after acclimatization. This protocol may find application in Citrus genetic improvement programs*.

***Keywords*:** *Citrus clementina*, *Cotyledons*, *Direct Organogenesis*, *Plant Regeneration*, *Tissue Culture*

1. Introduction

Citrus clementina Hort. ex Tanaka is a very important crop because of its excellent organoleptic and easy-peeling qualities. In *C. clementina*, as in *Citrus* spp., bud mutations arise spontaneously [1-4] and growers select them to improve horticultural traits of economically interesting species; there is also a high degree of interspecific sexual compatibility, so clementine is highly used in genetic improvement programs as mother plant to obtain new genotypes through hybridization by controlled inbreeding.

In vitro technique is a useful method to obtain true-to-type regenerated plants [5,6] as well as to induce somatic mutations [7] affecting characters of agronomic interest in order to develop new and improved genotype lines. There are several studies on plant regeneration by organogenesis and embryogenesis from different types of explants in *Citrus* genus [8-13], but very little information is available about procedures for achieving regeneration from clementine mandarin: embryos were induced from calli of ovular tissue [14], aberrant plants were obtained by direct and indirect somatic embryogenesis from the nucellus of eight cultivars [7], and gynogenesis [15] and androgenesis [16-18] were studied in different cultivars.

Cotyledons have high potential of regeneration [19,20] and represent a good source of tissue cultures. Organogenesis from cotyledons was successfully obtained in *Pongamia pinnata* (L.) [21], *Glicine max* (L.) Merril [22], *Dalbergia sissoo* Roxb. [23], *Capsicum annum* L. [24] and *Citrullus lanatus* (Thunb.) Matsum. & Nakai [25]. Beyond, in the last decade cotyledons and cotyledonary nodal regions were used as target tissues for transformation mediated by *Agrobacterium* [20].

The aim of this work was to induce *in vitro* plant regeneration in *Citrus clementina* to be used in *Citrus* genetic improvement programs. Three cultivars were investigated in the experiments and cotyledons were used as explants.

2. Materials and Methods

Ripe fruits of *C. clementina* "Monreal", "SRA 63" and

"SRA 64" of the germplasm collection belonging to the Istituto di Genetica Vegetale (sezione di Palermo), National Research Council (CNR) of Italy, were sampled at the beginning of December. The seeds, derived from open pollination, were drawn aseptically from the fruits into a laminar air flow and deprived of two teguments and of the embryo axis with a scalpel and forceps. Entire cotyledons (EC) and transversely cut cotyledons (CC) were used as explants. EC has one cut surface only, while CC were obtained cutting the cotyledon into three segments: the segment close to the embryo axis, identified as proximal (CCp), and the one next to it, called middle (CCm), have two cut sides each, while the farthest segment is the distal (CCd) and has one cut side. EC and CC were cultured on Murashige and Skoog [26] solidified medium containing vitamins, 500 $mg \cdot l^{-1}$ malt extract and 50 $g \cdot l^{-1}$ sucrose as basal medium. Three different concentrations of 6-benzylaminopurine (BAP) were tested in the basal medium: 8.8 μM, 13.2 μM and 17.6 μM. The pH of the medium was adjusted to 5.7 with 1 N KOH and the medium was autoclaved at 103 kPa (121˚C) for 20 min. Shoots about 5 mm long were isolated from cotyledons and transferred into culture tubes containing a hormone-free medium. Preliminary experiments were performed in the same culture conditions using seeds of fruits collected in the middle of November. All explants were incubated in a climate chamber at 26˚C ± 1˚C under 16-h photoperiod. Rooted plants were transferred into Jiffy 7® peat pellets to the green house and, when the radical apparatus was formed, they were potted and slowly submitted to acclimatization. Experiments were performed with 10 seeds per treatment and repeated thrice. The regeneration frequency (RF) (number of explants producing buds or shoots per total number of explants cultured multiplied 100), the number of buds/shoots per explant and shoot elongation were measured at 30, 60 and 90 days of culture.

Statistical Analysis

The statistical analysis was carried out at first through a descriptive analysis in which the data were presented as a mean value with its relative standard error by using Microsoft Office Excel 2003.

Following, the distributions of regeneration frequency, number of buds/shoots per explant and shoot elongation have been studied in order to properly specify a statistical model able to relate these dependent variables to the three explanatory variables BAP concentration, explant type and genotype.

BAP concentration was considered as a continuous variable while explant type and genotype were considered as factor variables with respectively four (EC, CCd, CCm, CCp) and three ("Monreal", "SRA 63", "SRA 64") levels.

The relationship between dependent and explanatory variables was modelled by using three different Generalized Linear Models (GLM) [27] according to the distribution of the response variables considered. In particular, in the regeneration frequency case, being a dichotomous variable (0 = No regeneration, 1 = regeneration), a binomial GLM was fitted; number of buds/shoots per explant was modelled through a Poisson GLM as it is a counting variable and finally shoot elongation was modelled by a Gamma GLM as its distribution showed a marked positive skewness. The best three GLMs resulted from a model selection procedure based on the Akaike Information Criterion (AIC) [28].

The statistical modelling has been carried out using R (R Development Core Team 2005) [29], a public domain statistical environment freely downloadable from the URL www.R-project.org.

3. Results and Discussion

Shoots began to emerge directly from the explants after three weeks of incubation and no callus around the emerging shoots was observed under the stereo microscope (**Figure 1(a)**). Callusing alone occurred very rarely and indirect organogenesis was never observed. The shoots in formation appeared as clusters of green protuberances that successively differentiated into buds (**Figure 1(b)**). They arose more frequently from the cut sides (**Figure 1(c)**) in both EC and CC and rarely directly from the intact cotyledon surface. Regeneration in all genotypes only occurred in the presence of BAP (**Figure 2**), that has a significant effect ($p \leq 0.001$) on the regeneration probability (**Table 1(A)**), while neither swelling nor morphogenic responses were noted when using the hormone-free basal medium. All BAP concentrations induced morphogenesis in "Monreal" and "SRA 64", whereas in "SRA 63" only the lowest level of BAP (8.8 μM) in EC and the highest concentration (17.6 μM) in the proximal segments were not inductive after 90 culture days (**Figure 2**). The young differentiated shoots transferred to the basal medium without growth regulators rooted easily and put new leaves, showing a regular growth (**Figure 1(d)**). The plantlets survived to the transfer to Jiffy 7® peat pellets into the green house and were acclimatized successfully after having been potted (**Figure 1(e)**). The entire process, from shoot emergence to plant acclimatation, was accomplished approximately in four-six months.

"Monreal" (**Figure 2(a)**) was the most reactive and fastest cultivar in terms of RF but only for EC explants: after 30 incubation days the RF was 33.33 ± 2.22 and 41.48 ± 1.48 respectively in 13.2 μM and 17.6 μM of BAP and an increasing trend to raise hormone concentration

was observed. The best regenerative potential was obtained by EC in 17.6 μM (50% ± 2.89% RF) after 60 incubation days. Proximal, middle and distal segments morphogenically responded in an unremarkably different way to BAP treatment.

In "SRA 63" (**Figure 2(b)**) the best BAP concentration for all explants was 13.2 μM and the most regenerative were EC and the middle segments (in both 33.33% ± 3.33% RF); these regeneration percentages were reached after 60 incubation days and held steady in both explants after 90 days. A slightly lower regenerative response was obtained in the distal segments in 13.2 μM of BAP (26.67% ± 3.33%), while 17.6 μM concentration induced poor organogenesis, ranging from 6.67% ± 1.67% (distal segment) to 13.33 ± 3.33 (EC and middle segment) after 90 days of incubation.

In "SRA 64" (**Figure 2(c)**) the best regeneration occurred in EC in BAP 13.2 μM (30.04% ± 3.92% RF) after 90 incubation days. Regarding CC, the best morphogenetic response was obtained in the distal segments in BAP 13.2 μM (21.85 ± 0.37 RF) and in the middle segments in both 8.8 and 13.2 μM (18.52 ± 3.70) concentrations after 90 incubation days. Regeneration was very low in proximal segments, ranging from 3.70 ± 1.85 (BAP 17.6 μM) to 7.04 ± 1.48 (BAP 13.2 μM).

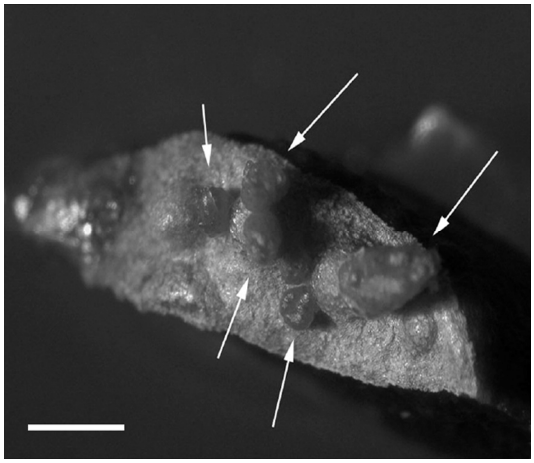
The probability of regeneration (**Table 1(A)**) is different in the three genotypes: "SRA 64" (p_0.001) had a probability of regeneration lower than the "Monreal", while the probability of regeneration for the "SRA 63" (p_0.05) resulted marginally different from the "Monreal". Beyond, the probability of regeneration for the entire cotyledon was significantly higher than the other three explant types (**Table 1(A)**).

The number of buds/shoots differentiated per explant after 60 days of incubation (**Table 2**) ranged from 1 to 4.28 ± 1.37 in all cultivars. Only the explant types resulted significant (**Table 1(B)**) so the other two variables (BAP concentration and genotype) were eliminated from the model in the **Table 1(B)**. Significative differences in the number of buds/shoots per explant variable were achieved between the entire cotyledon and the distal ($p \leq 0.001$) and medium ($p \leq 0.05$) segments; on the contrary entire cotyledon and proximal segment are not significatively different between them and they were the explants with the highest number of buds/shoots differentiated per explant.

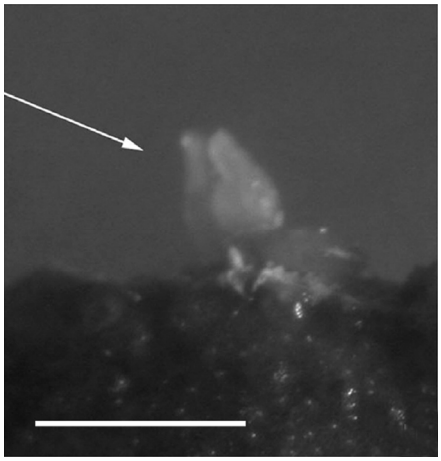
The shoots generated from EC explants (**Table 2**) was always significatively much longer ($p \leq 0.001$) than the

Table 1. Akaike Information Criterion statistical procedure on the regeneration frequency variable (A), the number of buds/shoots per explant variable (B) and the shoot elongation variable (C) in *Citrus clementina* cultivars, estimated respectively from a GLM-binomial, GLM-Poisson and GLMGamma (link Identity). Intercept 1 represents "Monreal", hormone-free medium and entire cotyledon; intercept 2 and 3 represent entire cotyledon; 6-benzylaminopurine (BAP); transversely cut cotyledons distal (CCd), middle (CCm) and proximal (CCp) segments. Significance codes: '*' p ≤ 0.001; '**' p ≤ 0.01; "*" p ≤ 0.05; "." p ≤ 0.1; " " p ≤ 1. Standard error (SE).**

			Estimate	SE	z-	p-value
A	**Regeneration Frequency variable**	Intercept 1	–2.366	0.296	–7.988	1.37e -15
		BAP	0.113	0.018	6.129	8.82e-10
		"SRA 63"	–0.453	0.247	–1.830	0.067
		"SRA 64"	–0.960	0.228	–4.214	2.50e-05
		CCd	–0.728	0.257	–2.827	0.004**
		CCm	–0.574	0.249	–2.304	0.021*
		CCp	–1.047	0.278	–3.763	0.0001***
B	**Number of buds/shoots per explant variable**	Intercept 2	1.247	0.074	16.780	<2e-16***
		CCd	–0.505	0.146	–3.454	0.0005***
		CCm	–0.324	0.132	–2.451	0.014*
		CCp	0.005	0.136	0.040	0.967
C	**Shoot elongation variable**	Intercept 3	5.117	0.560	9.131	9.68e-16
		CCd	–2.394	0.684	–3.498	0.0006***
		CCm	–3.1549	0.621	–5.082	1.24e-06***
		CCp	–2.612	0.701	–3.725	0.0002***



(a)



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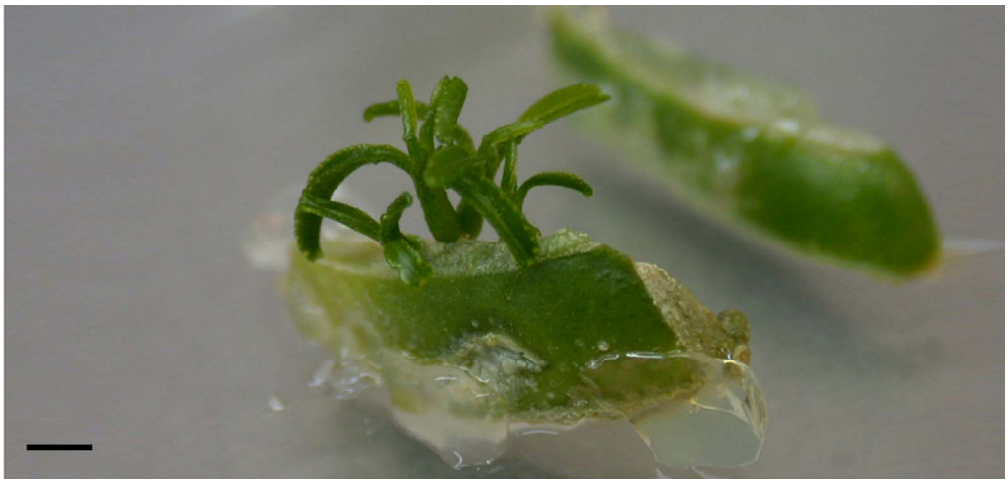

(c)

(d)

(e)

Figure 1. Different development stages in the plantlet formation via direct organogenesis from cotyledons in *Citrus clementina* "Monreal"; the same pattern occurred in "SRA 63" and "SRA 64". (a) Regeneration appeared with clusters of green swelling and protuberances (arrows); (b) Differentiation of bud (arrow); (c) Shoots arising from the wounded side; (d) Shoot transferred in the hormone-free basal medium showing a regular growth; (e) Potted plant established *in vivo* conditions. Bars = 1 mm.

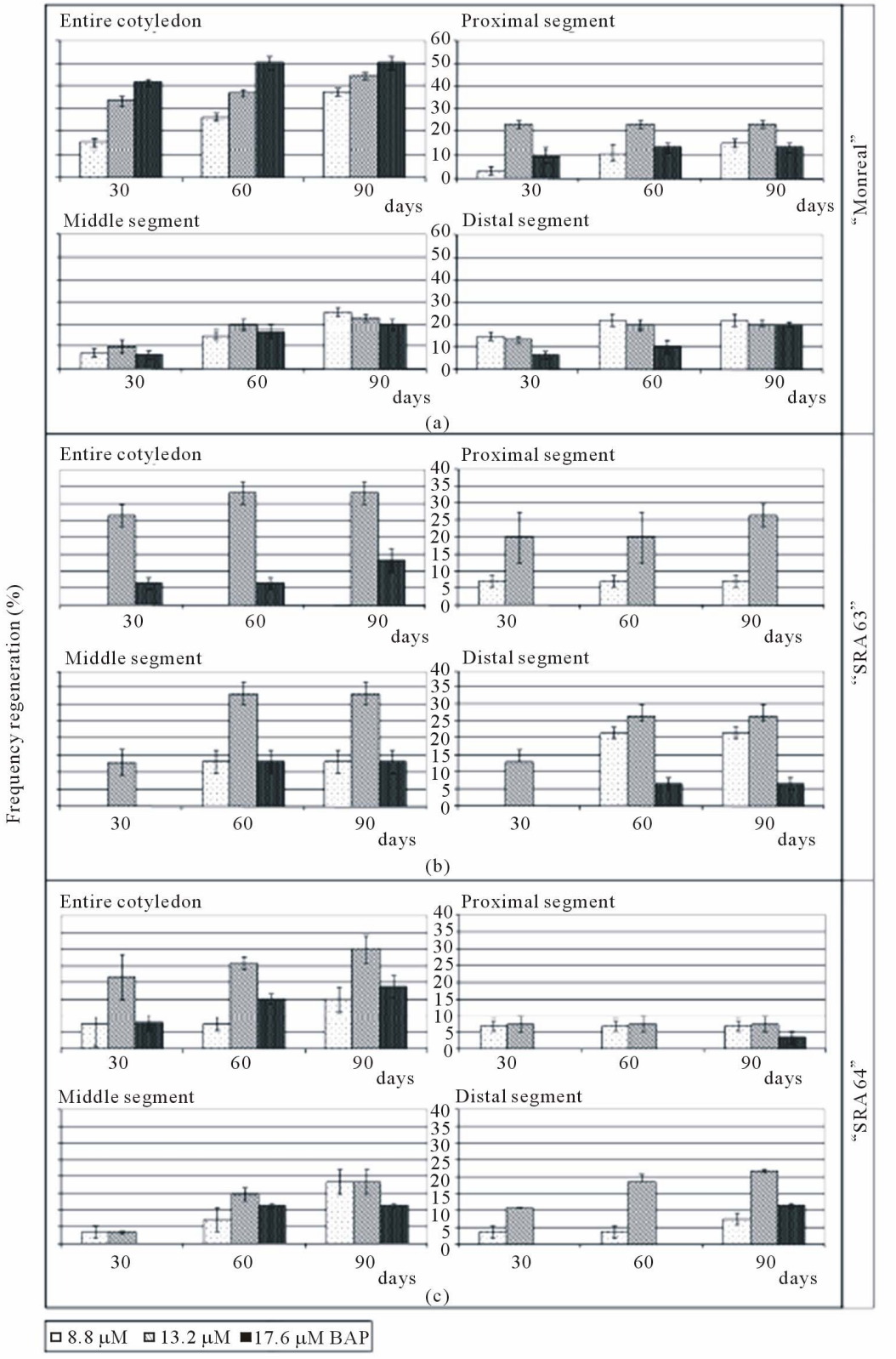

Figure 2. Effect of BAP concentration on regeneration frequency (%) from entire and transversely cut cotyledons (proximal, middle and distal segments) of *Citrus clementina* cultivars, "Monreal" (a) "SRA 63" (b) and "SRA 64" (c), at 30, 60 and 90 days of incubation. Vertical bars represent standard error of the means.

Table 2. Influence of BAP concentration on number of buds/shoots differentiated per explant by direct organogenesis and the shoots' length (mm) after 60 days of incubation in "Monreal", "SRA 63" and "SRA 64" *Citrus clementina* cultivars, in entire cotyledons (EC) and transversely cut cotyledons (CC), proximal (p), middle (m) and distal (d) segments. Mean ± Standard error.

Clementine Cultivars	BAP (μM)	Number of Buds/Shoots per Explant				Length of Shoots			
		EC	CC			EC	CC		
			p	m	d		p	m	d
	8.8	3.14 ± 0.79	1.66 ± 0.33	2.75 ± 1.10	2.66 ± 0.91	3.82 ± 0.88	2.66±0.66	2.50 ± 1.19	2.20 ± 0.35
"Monreal"	13.2	4.27 ± 1.18	4.28 ± 1.37	2.50 ± 0.26	2.50 ± 0.95	5.20 ± 1.16	2.68±0.60	2.38 ± 0.57	3.75 ± 1.86
	17.6	3.6 ± 0.68	3.25 ± 1.10	2.40 ± 0.60	2 ± 0.50	5.98 ± 1.24	3.95±2.05	1.60 ± 0. 40	1 ± 0
	8.8	0	7	3.50 ± 1.50	1 ± 0	−	2	1.25 ± 0.25	1.33 ± 0.33
"SRA 63"	13.2	4.2 ± 1.11	2.66 ± 0.33	3.20 ± 0.91	2.25 ± 0.62	6.44 ± 2.34	2±0	2.20 ± 0.84	2.62 ± 1.46
	17.6	4	0	1	1	7	−	2 ± 0	7
	8.8	2.5 ± 0.5	3.50 ± 1.50	2.50 ± 1.50	1	2 ± 0	1 ± 0	1.90 ± 0.1	4
"SRA 64"	13.2	3.28 ± 0.71	3.50 ± 1.50	2.50 ± 0.86	2 ± 0.77	4.44 ± 1.02	1.25 ± 0.25	1.75 ± 0.47	3.3 ± 0.7
	17.6	1.25 ± 0.25	0	1.66 ± 0.66	1	4.5 ± 2.02	−	1.33 ± 0.33	1

three types of CC ones (**Table 1(C)**) and, beyond, the shoot elongation was not influenced significantly by the BAP concentration and the genotype. The transfer of longer shoots to fresh medium has more survival possibility than shorter ones (data not shown).

In previous and preliminary experiments cotyledons of seeds coming from fruits collected in November showed a similar pattern of morphogenic response with reference to the influence of BAP in comparison to seeds of December: 17.6 μM BAP for "Monreal" and 13.2 μM BAP for "SRA 63" and "SRA 64" were the best concentrations. Instead the regeneration frequency was higher (80% RF in "Monreal", 75% RF in "SRA 63" and 60% in "SRA 64") and occurred better in CC than EC. This behavior may be explained by the fact that cells belonging to juvenile plant material have a higher regeneration competence and more rapid rates of proliferation in tissue cultures if compared to explants collected from mature tissue [8,30]. It should be noted that the regeneration frequency related to the explant physiological state depends on the species (species-specific), as indeed the mature cotyledons in soybean (*Glycine max* (L.) Merr.), for example, were observed to be more regenerative than immature ones [20]. The morphogenic inducting effect of wounds [31] may be more productive in younger tissues, making the CC explants more regenerative than EC.

In the present study, it was demonstrated that BAP is absolutely required to induce *in vitro* morphogenesis in clementine. In *Citrus*, the cytokinin 6-benzylaminopurine has been reported in a large number of protocols as promoting the formation of adventitious buds or shoots [10,32-34]. Its use could reduce the risk of somaclonal variability in regenerants as opposed to diphenylurea derivates like N-(2-chloro-4-pyridyl)-N'-phenylurea that induces high levels of somaclonal variability [35]. The frequency of regeneration increased with the permanence in the medium supplemented with BAP, demonstrating that cells maintained their organogenic competence during the 90 incubation days.

Regeneration from cotyledon explants has been reported in several taxa [21-24,36,37] but it has been recorded in only a few species belonging to *Citrus* genus: adventive embryos formation in *Citrus reticulata* Blanco (Nagpur mandarin) and *C. jambhiri* Lush. (Rough lemon) [5], indirect somatic embryogenesis in *C. reticulata* "Local Sangtra" [38] and indirect shoot regeneration in *C. grandis* (L.) Osbeck (pummelo) [32,39].

4. Conclusions

Shoot regeneration in *Citrus clementina* can be obtained through direct organogenesis using cotyledons as explant and 6-benzylaminopurine growth regulator is essential to induce differentiation. The best BAP concentration for inducing regeneration has been determined as 17.6 μM BAP for "Monreal" and 13.2 μM BAP for "SRA 63" and "SRA 64" cultivars. The entire cotyledons were always more regenerative than transversely cut cotyledons for all the three cultivars, but a different morphogenic response was observed among the tested genotypes, showing "Monreal" having the highest organogenic potential.

This protocol may find application in *Citrus* genetic improvement and in studies concerning the achievement

of new hybrids reducing the propagation time in respect to conventional methods. The obtained progeny, hybrid and heterogeneous, can be multiplied through direct adventitious shoot organogenesis, thus obtaining more identical individuals per seed who are more genetically stable in comparison to plants regenerated via callus, in which the presence of somaclonal variability is more probable. The plantlets, that can be transferred successfully *in vivo* in the greenhouse, may be monitored and evaluated for all new agronomic characteristics**.**

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