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Micropropagation from cotyledonary explants of grapefruit (*Citrus paradisi* Macf.) by indirect and direct organogenesis

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Abstract

Grapefruit (*Citrus paradisi* Macf.) is an economic fruit crop worldwide cultivated for its nutritional value, and aromatic flavours. However, the development of new varieties and high production of quality seedling constitutes a limit to improving grapefruit yields. An efficient regeneration protocol could be of great interest for grapefruit transformation, micropropagation, and germplasm conservation. *In vitro* plant regeneration systems of *Citrus paradisi* have been studied by using cotyledonary explants. Different concentrations of plant growth regulators were used to explore the organogenic potential of cotyledonary explants under laboratory conditions. For indirect organogenesis using cotyledon segment explants, a low concentration of2,4-dichlorophenoxyacetic acid (2,4-D) lead to the formation of white callus, while a high concentration of 2,4-D induced brown callus and adventitious root differentiation. The best response for callogenesis was obtained on Murashige and Skoog (MS) medium supplemented with 2 mg/L of 2,4-D. Only brown callus cultured on MS added with 2 mg/L 6-benzylaminopurine (BAP) has regenerated shoots, but the percentage of callus producing shoots was very low (6.66 ± 2.68%). Regarding direct organogenesis from the cotyledonary node explants, the best response for shoot proliferation was observed on MS supplemented with 4 mg/L BAP (86.66 ± 12.58% of explants regenerating shoots, and an average number of 5.45 ± 0.45 shoots per explant). Low percentages of *In vitro* rooting of regenerated shoots were observed with different concentrations of α-naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA). Nevertheless, the highest rooting percentage (13.33 ± 2.88%) was obtained on MS containing 2 mg/L NAA. These results provide new information for mass regeneration and the production of healthy seedlings, and can be applied in grapefruit improvement programs.

1. Introduction

Cultivated citrus is one of the most important fruit crops in the world, which is widely distributed across more than 140 countries mainly in tropical and subtropical areas [1]. The genus *Citrus* belong to the angiosperm subfamily Aurantioideae of the Rutaceae family which includes many cultivated species, (e.g. " sweet orange" *Citrus sinensis*; "mandarin" *Citrus reticulata*, "grapefruit" *Citrus paradisi*, "lemon" *Citrus limon* and "pomelo" *Citrus grandis*), which are essentially used for food industry, due to their fine flavour and medicinal properties [2]. According to the Food and Agriculture Organization of the United Nations (FAO), in 2019 the world production of citrus fruits was estimated at more than 143 million metric tonnes [3]. *Citrus paradisi* is a natural stabilized hybrid between a pomelo and sweet orange [4]. Grapefruit is popular and cultivated for its nutritional value, aromatic flavours [5], and bioactive compounds contents such as flavonoids [6,7]. In many countries, grapefruit is used as antimicrobial, anti-inflammatory, antioxidant and also for cancer prevention or heart health maintenance [8].

Despite their economic importance and extensive cultivation, grapefruit trees and fruits still have several problems including insects by pests, diseases, post-harvest losses, abiotic stress etc. that can significantly reduce yield components.

Thus, the development of improved varieties and high production of quality seedling are imperative to overcome these problems and increase yields. Like others citrus crops, improvement of grapefruit by conventional breeding methods is hampered by many barriers related to plant biology, nucellar polyembryony (somatic and zygotic embryos), self-incompatibility, inter-incompatibility, slow growth and a long juvenile period [9,10]. However, advances in plant biotechnology provide new opportunities allowing to avoid these natural barriers [11,12]. *In vitro* plant regeneration systems can be used for the mass production disease free plants and as a first step for introduction of genetic variations on citrus crops [13–15].

In comparison to most other citrus crops very few studies are available regarding *in vitro* shoot regeneration of *C. paradisi*. Adventitious leafy shoots were obtained from internode [16] and epicotyl [2,17–21] explants of *C. paradisi*. Moreover, the establishment of efficient i*n vitro* regeneration protocol for a given species requires, the investigation of organogenic potentialities of each explant. Among other citrus species, cotyledon or cotyledonary node explants have been successfully used to establish an efficient protocol for *in vitro* regeneration of *C. sinensis* [22], "siam orange" *C. nobilis* [23], *C. reticulata* [14], "mexican lime" *C. aurantifolia* [24], *C. jambhiri* [25] and *C. tangerina* [26]. These explants were also used in another economic crops such as "mango" *Mangifera indica* [27], "sesame" *Sesamum indicum* [28], and "butter fruit" *Dacryodes edulis* [29,30]. This work aims to study the potentialities of cotyledonary explants for rapid *in vitro* propagation of *C. paradisis*, which

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can be used in genetic improvement programs. More specially, to access the effect of plant growth hormones on the indirect and direct organogenesis from cotyledonary explants.

2. Materials and Methods

2.1. Plant material and preparation of explants

Ripe fruits of *C. paradisi* var. White Marsh were harvested from 8 years old trees in agro-ecological zone IV of Cameroon (Littoral Region, Moungo Division). Fruits were thoroughly washed with running tap water and disinfected by soaking for 5 min in 95% ethanol. Subsequently, into a laminar airflow, the fruits were flamed and dissected with a scalpel for seed extraction. Seeds without teguments were used firstly, to obtain cotyledon explants after removing the embryo axis according to the protocol previously described [25]. On the other hand, seeds were cultured on half MS medium for 30 days to obtain cotyledonary node explants from seedlings developed as previously reported [14].

2.2. Experimental design and culture conditions

All the experiments were conducted under controlled conditions at the *in vitro* laboratory of Centre Africain de Recherche sur Bananiers et Plantains (CARBAP). The experimental design was set up in a randomized complete block with three replications. The basic culture medium was Murashige & Skoog (1962) (MS) with 3% sucrose and 2 mg/L vitamins, adjusted to pH 5.8 with 1 N NaOH and solidified with 2.5 g/L phytagel. The medium was sterilized by autoclaving at 120 ºC (103 kPa) for 20 min. The cultures were maintained at 26±2 ºC with a photoperiod 16h/8h (day/night) provided by cool white fluorescent light, and 70-80% of relative humidity.

2.3. Callogenesis and shoot proliferation by indirect organogenesis

Callogenesis

Cotyledon explants of 0.49 to 1 cm^2 were cultured on MS basal medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5, 1, 1.5, and 2 mg/L). MS medium without plant growth regulators was used as a control. Each treatment was tested with 20 explants per replication. After 30 days, the percentage of explants producing callus and callus characteristics (colour, size, texture, and presence or absence of root differentiation) were recorded.

Shoot proliferation

The ability of callus to regenerate adventitious buds was investigated by transferring callus on MS medium supplemented with different concentrations of 6-benzylaminopurine (BAP) (1, 2, 3, 4 mg/L). MS medium without plant growth regulators was used as a control. Each treatment was tested with 20 explants per replication. The percentage of budded callus was recorded after 40 days of culture.

2.4. Shoot proliferation and rooting by direct organogenesis

Shoot proliferation from the cotyledonary node explants

The seeds were germinated aseptically on half MS basal medium without growth regulators. Cotyledonary explants were excised from 30 days old seedlings and cultured on MS medium containing BAP at different concentrations (1, 2, 3, 4 mg/L). MS medium without cytokinin was used as a control. Each treatment was tried with ten explants per replication. After 45 days, the percentage of explants budded and proliferated, the number of shoots per explant and shoot length were recorded.

In vitro rooting of shoots

Leafy shoots (more than 1.5 cm of length) derived from cotyledonary node explant of *C. paradisi* were separated and individually cultured in a vertical position. MS medium supplemented with different concentrations of α-naphthalene acetic acid (NAA at 2 and 4 mg/L) or indole-3-butyric acid (IBA at 1 and 2 mg/L) were used to induce *in vitro* root formation. MS medium without auxin was used as a control. Each treatment was tried with five explants per replication. The percentage of shoots inducing roots and the average number of roots per shoot were recorded after 30 days of culture.

2.4. Statistical analyses

Data analyses were performed with R script software. For each evaluated parameter, normality was checked with Kolmogorov-Smirnov test. The data were subjected to one-way analysis of variance (ANOVA or Kruskal-Wallis) with 5 % significance level to identify the variation within the treatments. The comparisons between the treatments were carried out by using Duncan's test [31] or Kruskal's test (for data with an anormal distribution). The graphical representations of the data were carried out by using R script software.

3. Results

3.1. Effects of hormonal treatments on indirect organogenesis

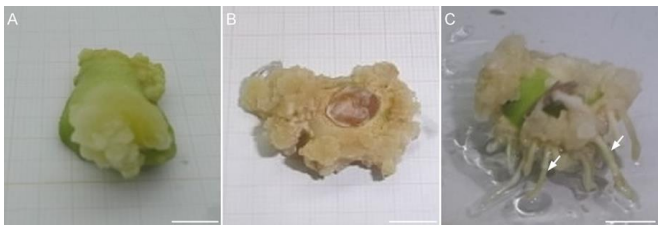
Effects of 2,4-D on callogenesis

Callus differentiation from cotyledon explants was significantly ($p < 0.001$) influenced by 2,4-D concentration in the culture medium (Table I). Callus exhibited a friable texture, and brown or white colour (Figures 1A and 1B). High concentrations (1.5 or 2 mg/L) of 2,4-D induced mainly brown friable callus with some adventitious roots (Figure 1C). While low concentrations (0.5 or 1 mg/L) induced white friable callus without adventitious roots. The percentage of explants producing callus increased with 2,4-D concentration in the culture medium. MS medium supplemented with 2 mg/L 2,4-D, showed the highest response of callogenesis (Table I).

Table 1: Effect of 2,4-D concentration on callus induction from cotyledon explants of *C. paradisi*

2,4-D Concentration (mg/L)	Callus characteristics				Percentage of explants producing callus (%)
	Texture	Colour	Size (cm)	Adventitious roots	
0	-	-	-	-	0
0.5	Friable	White	0.5-1	No differentiated	45 ± 5c
1	Friable	Brown or white	1-1.5	No differentiated	56.66 ± 7.63bc
1.5	Friable	Brown	3-3.5	Differentiated	68.33 ± 7.63b
2	Friable	Brown	3-3.5	Differentiated	83.33 ± 10.4a

Data were recorded after 30 days of culture. 0 mg/L of 2,4-D represents the control explants. The values in each column followed by the same letter are not statistically different using Duncan's test at $p<0.05$.



A: white friable callus; B: brown friable callus; C: callus exhibited adventitious roots. Arrows indicate adventitious roots. Scale bar = 1 cm.

Figure 1: Callus produced from cotyledon explants of *C. paradisi* on MS medium supplemented with 2,4-D after 30 days of culture.

Effect of BAP on shoot proliferation from callus

Adventitious budding depends on callus colour and BAP concentration in the culture medium. No response was recorded on withe callus. However, on MS medium supplemented with 2 mg/L of BAP, brown callus changed to green colour and induced the formation of adventitious shoots (Figure

SI). Nevertheless, only 6.66 ± 2.68 % of callus were able to regenerate shoots after 40 days of culture.

3.2. Effects of hormonal treatments on direct organogenesis

Effect of BAP on shoot proliferation from cotyledonary nodes

Seed germination began 2 days after culture and the highest germination percentage was 97% after 12 days (Figure S2). The cotyledonary node explants were excised from the seedlings and cultured in a shoot proliferation medium (Figure 2A).

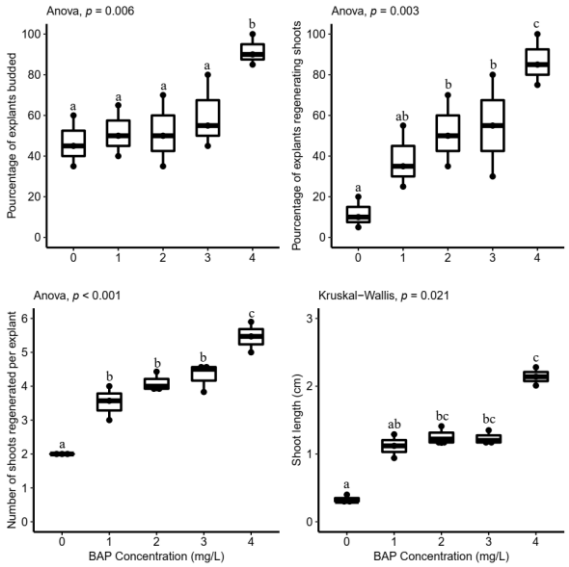
In vitro regeneration of *C. paradisi* via cotyledonary node explant showed that the use of exogenous growth regulators is not essential for the proliferation of leafy shoots, but their use significantly improves shoot proliferation. The percentage of budded explants and proliferated explants, the number of shoots regenerated per explant as well as the growth of shoots increased when BAP was added to the culture medium (Figure 3). The variation of BAP concentration affected significantly the response of the explant for shoot proliferation after forty-five days of culture. The best BAP treatment for all parameters evaluated was 4 mg/L with 86.66 ± 12.58% of explants regenerating shoots and an average of 5.45 ± 0.45 shoots per explant (Figures 2B and C).

A: cotyledonary node explant cultured; B: axillary buds induced; C: Shoot proliferation obtained on MS added with 4 mg/L of BAP; D: In vitro rooting shoot on MS added with 2 mg/L of NAA. Ax= axillary buds; Tm= tumours. Scale bar = 1 cm.

Figure 2: *In vitro* plant regeneration from cotyledonary node explants of C. paradisi

Effects of AIB and NAA on shoots rooting

Leafy shoots were transferred on MS medium supplemented with plant growth regulators for rooting. We found that the type and variation of auxin concentration affects significantly ($P < 0.01$) the rooting potential of the leafy shoots. No rooted shoot was recorded on the control medium. Increase rooting for regenerated shoots of *C. paradisi* requires the presence of auxin in the culture medium, but the rooting percentage obtained was very low. Only the treatments at 2 mg/L NAA and 1 mg/L AIB have induced root differentiation, with a rooting percentage of 13.33 ± 2.88% and 5% respectively (Table 2, Figure 2D). Auxin treatments also led to the development of tumours at the base of the shoot.



Data were recorded after 45 days of culture. 0 mg/L of BAP represents the control treatment. The values in each graph followed by the same letter are not statistically different using Duncan's test or Kruskal's test at $p < 0.05$.

Figure 3: Effect of BAP concentration on shoot proliferation from cotyledonary node explant of *C. paradisi*.

Table 2: Effect of auxin treatments on in vitro rooting of regenerated shoots of *C. paradisi*.

Plant growth regulators	Concentration (mg/L)	Percentage of shoots inducing roots (%)	Average number of roots induced per shoot
Control	0	0	0
IBA	1	5.00 ± 0.00b	1.00 ± 0.00b
	2	0	0
NAA	2	13.33 ± 2.88a	1.33 ± 0.57a
	4	0	0

Data were recorded after 30 days of culture. The values in each column followed by the same letter are not statistically different by Duncan's test at $p < 0.05$.

4. Discussion

4.1. Indirect organogenesis

The variation of callus characteristics would be result from interactions between endogenous factors such as the level of growth hormones within explant and exogenous hormones levels [32]. However, the mechanisms remain poorly understood. Different effects of 2,4-D on callogenesis from leaf fragments explants have also been reported in *C. grandis* [33]. However, callus with identical characteristics have been obtained from cotyledon explants of *C. jambhiri* [34]. Adventitious roots differentiation would be due to a double function of 2,4-D at the high concentrations. Indeed, 2,4-D is an auxin involved not only in division and lengthening cell, but also in vascular tissues and roots differentiation [35]. The formation of adventitious roots was induced on cotyledon callus of *C. jambhiri* cultured on MS medium added with 1.5 mg/L of 2,4-D [36]. The absence of callogenesis response observed on MS medium without plant growth regulators, indicates that the endogenous auxin content in the cotyledon explants is not sufficient to induce callus formation. Similar results were observed with cotyledon explants of *C. jambhiri* [34]. On the other hand, 2,4-D is not essential for callus induction from ovule or style explants of *C. paradisi*, but their use improves significantly the rate of callogenesis [37].

Plant regeneration via shoot organogenesis from callus cultures was difficult. We found that white callus is not able to regenerate adventitious

shoots. This absence of organogenesis response suggests that, white callus is composed by non-embryonic or non organogenic cells. It was reported that, the white callus obtained from ovule explants of *C. paradisi* are not able to regenerate shoots on Murashige and Tucker basal medium supplemented with different combinations of plant growth regulators concentrations [37]. Regarding the brown callus, low shoot regeneration rate obtained, suggest the presence of embryonic cells, but we need to test a wide range of cytokinin concentrations for improve shoot regeneration from callus.

4.2. Direct organogenesis

We observed shoot regeneration from cotyledonary node explant of *C. paradisi* on MS medium without cytokinin, suggesting the endogenous hormone content would be sufficient to allow direct organogenesis. Similar results were also reported from epicotyl explants of *C. paradisi* [18]. Contrariwise, cotyledonary node explant of *C. reticulata* could not induced shoot proliferation on MS medium without plant growth regulators [14]. In fact, ability of explant for shoot proliferation depends strongly to plant genotype [38].

The presence of BAP on MS medium increases significantly shoot proliferation. Improving of shoot proliferation due to BAP treatments could be linked to the ability of cytokinin to stimulate or intensify the synthesis of nucleic acids and proteins necessary for organogenesis [39]. A regeneration rate of 68% and an average of 3.96 leafy shoots per explant of *C. paradisi* were obtained on MS medium supplemented with 2 mg/L BAP [18]. Positive effects of BAP on shoot proliferation have been previously reported in *Citrus* sp. Shoot organogenesis from cotyledonary node of *C. reticulata* increases with the presence of cytokinin, and the highest regeneration response (84% proliferation rate and an average number of 6.45 shoots) was obtained on MS added with 4 mg/L BAP. In *C. aurantium*, 63 % of nodal explants proliferated with an average of 5 shoots per explant in presence of 2 mg/L BAP [40]. An average number of 7.8 shoots per explant from epicotyl segments of *C. reticulata* was obtained on MS modified with 2 mg/L BAP [41]. However, in other cases culture medium containing BAP requires combination with another plant growth regulators to improve shoot proliferation. It was reported that, 71 % of the cotyledonary node explants of *C. tangerina* produced 5.6 shoots per explant in presence of 8.88 µM BAP and 0.54 µM NAA [26]. Shoot tip explants of *C. reticulata* cultured in MS media supplemented with 1 mg /L BAP and 1.5 mg /L Kinetin (Kn) showed highest shoot percentage (84%) with 7.99 shoots per explant [42]. Nodal segments explants of *C. aurantium* produced 7.4 shoots per node on 1 mg/L BAP and 0.5 mg/L Kn [40].

Generally, *in vitro* rooting of shoots in *Citrus* sp. requires auxin treatments, suggesting that hormonal balance (cytokinin-auxin ratio) of regenerated shoots would not be favourable for root differentiation. Exogenous plant growth regulators treatment is essential for the rooting of leafy shoots in *Citrus* sp. [13]**.** These observations were reported in *C. reticulata* [14], *C. aurantium* [40], and *C. jambhiri* [34]. We found that low concentrations of auxin (NAA or IBA) can induce root formation, but the rooting percentage was very low. Previous studies **r**eported that, rooting of shoots can be a major barrier to *in vitro* regeneration of plants on *Citrus* genus. A rooting percentage of 3.2% for leafy shoots of *C. sinensis* was achieved on MS medium supplemented with 3 mg/L NAA after three months of culture [43]. Low rates of rooting were also observed in shoots of Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) cultured on medium containing 5.4 µM NAA [44]. It should be noted that a high rooting percentage was reported in other studies. A good rate of rooted shoot of *C. paradisi* was obtained on MS medium added with 5 µM NAA [20,45]. 88 % of leaf shoot with 3 roots per shoot of *C. reticulata* in the presence of 2 mg/L NAA [14]. In *C. jambhiri* the best percentages of rooting for shoots of were obtained when NAA or AIB are supplemented on the culture medium [34]. These results suggest that, in our conditions we need to test different combination of auxins concentrations to improve *in vitro* rooting step. The formation of callus at the base of shoot, was also observed with leafy shoots of *C. sinensis* in presence of NAA [46].

5. Conclusion

Study of potentialities of cotyledonary explants of *C. paradisi* to indirect or direct organogenesis showed that, 2,4-D is essential for callus induction from cotyledon segments explants. However, callus obtained did not show efficient ability for indirect organogenesis, suggesting cotyledon explants is not a useful explant for *in vitro* multiplication of *C. paradisi*. Regarding direct organogenesis, plant regeneration was developed from cotyledonary node. Increasing of shoot regeneration in presence of BAP suggests that plant growth hormone had a great influence in shoot proliferation stage. For *in vitro* rooting of shoot, application of NAA at low concentration showed the best results. This *in vitro* regeneration protocol could be improved, to achieve high efficiency propagation of *C. paradisi*. Micropropagation of grapefruit could make it possible to obtain healthy inexpensive seedling, leading to improved agronomic yields.

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