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Effects of plant growth regulators on callogenesis, somatic embryogenesis and plantlet regeneration of *Melia azedarach* L.

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Abstract
We developed an efficient regeneration system through indirect somatic embryogenesis for *Melia azedarach* L. Different culture media with various plant growth regulator (PGR) combinations were evaluated. It was found that callus induction, proliferation and morphology depend strongly on the PGR combination of the culture medium. In fact, the callus induction rate varied from 7 to 100%. The highest callogenesis rate was observed on half-strength Murashige and Skoog (MS/2) medium supplemented with 3 mg/L 1-naphthalene acetic acid (NAA), 1 mg/L zeatin and 5 mg/L gibberellic acid (GA_3). Calli were either white and friable, light brown and hydrated or brown and compact, with different proliferation potential. Somatic embryo formation was observed either directly on the explant or on the previously induced calli, depending on the PGR combination of the induction medium. The highest somatic embryogenesis rate was 29% in the calli induced on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L 6-benzylaminopurine (BAP) and 5 mg/L GA_3. Callus induced on MS/2 medium containing 3 mg/L indole-3-butyric acid (IBA), 1 mg/L BAP and 5 mg/L GA_3 showed a somatic embryogenesis rate of 5% while a similar rate was observed on the explants cultured on MS/2 medium containing 3 mg/L NAA, 1 mg/L zeatin and 5 mg/L GA_3. However, in this latter case, somatic embryos were formed directly on the explant. After transferring somatic embryos to the germination medium, the germination rate was 51.28%. All germinated embryos were successfully converted into complete plantlets and showed normal growth and development.

Keywords: Callogenesis; *In vitro*; *Melia azedarach* L.; plant growth regulator; regeneration; somatic embryogenesis.

Introduction
Melia azedarach (L.), also knowns as White Sedar, is a plant species native to tropical Asia and widely distributed in south-east Asia, Persia, India and Australia [1, 10]. It belongs to the *Meliaceae* family and is considered as an ornamental plant in many countries, whereas in many others, including Morocco, *Melia azedarach* is considered as an invasive species.
In the beginning of the 20th century, during the great locust invasions, it was noticed that *Melia azedarach* was not attacked by locusts. Since then, studies have been carried out to investigate the pesticide properties of this plant species [3]. The first active ingredient of *Melia azedarach* was extracted and purified in 1946 [4]. Subsequently, numerous studies have revealed the presence of active ingredients with effective insecticidal properties. Today, *Melia azedarach* is considered highly effective against agricultural pests. This species is also considered as a very promising means to reduce the use of chemical products in agriculture, and a good tool in the biological control of agricultural pests. Thus, *Melia azedarach* could contribute in reducing the negative impacts of chemical pesticide that affect humans, livestock, environment, useful fauna and biodiversity.
Despite the high importance of *Melia azedarach*, few studies have been performed to develop efficient propagation techniques for it. On the other hand, in order to benefit from *Melia azedarach* properties, it is necessary to have sufficient plant material. *Melia azedarach* can be propagated by seeds and stem cuttings [24]. However, the use of these two conventional techniques has many limitations [18].
The use of *in vitro* culture techniques seems to be a very good alternative for rapid and large-scale propagation of *Melia azedarach*. In fact, *in vitro* culture techniques are becoming more

and more essential for the rapid and large-scale propagation, conservation and genetic improvement of plant species.

To date, few studies were published on *Melia azedarach* regeneration by *in vitro* techniques. Mroginski and Rey [24], Thakur *et al.* [18] and Ahmad *et al.* [2] developed regeneration systems for *Melia azedarach* through microcuttings. Vila *et al*. [19, 20, 22] succeeded regeneration of *Melia azedarach* through adventitious organogenesis while regeneration through somatic embryogenesis was reported from cotyledon explants and immature zygotic embryos by Deb [5], Sharry *et al*. [16, 17] and Vila *et al*. [21].

Among all the *in vitro* culture techniques used, somatic embryogenesis is the most interesting. In fact, somatic embryogenesis had high impacts in the development of many plant species and can be used for various biotechnological purposes. Somatic embryogenesis is the process by which somatic embryos are formed from the explants in culture. It comprises the following step: callus induction (in case of indirect somatic embryogenesis), somatic embryogenesis expression, somatic embryo germination and conversion into complete plantlets. Reports concerning the use of somatic embryogenesis for *Melia azedarach* were limited to few genotypes that belong to restricted areas of cultivation.

The purpose of the present work was to develop an efficient and reproducible regeneration system for a *Melia azedarach* genotype growing in Morocco. Thus, various plant growth regulator (PGR) combinations were assessed and their effects on callus morphology and somatic embryogenesis pattern (direct or indirect) were evaluated.

Material and methods

Plant material and disinfection

Mature fruits of *Melia azedarach* L. were collected from an adult tree located in the National School of Agriculture of Meknes (Morocco; 33°50'39.9"N 5°28'38.7"W). The fruits were thoroughly washed with tap water then the pulp and the endocarp were removed. Seeds were surface sterilized with a commercial bleach solution (10%) for 3 minutes followed by three rinses in sterile distilled water (10 min each). Afterwards, zygotic embryos were carefully extracted.

Culture medium

The culture medium used in the present study was that of Murashige and Skoog [15] at full strength (MS macro-elements, MS microelements and MS vitamins) or half-strength (MS/2 macro-elements, MS/2 microelements and MS/2 vitamins), depending on the culture phase. All culture media were solidified with agar (8 g/L) and their pH was set to 5.7.

Culture conditions

During the induction and expression experiments, all cultures were kept under total darkness. For germination and conversion of somatic embryos into complete plantlets, the embryos were maintained under a 16h photoperiod. In all experiments, the temperature was set at 25°C.

Somatic embryogenesis induction

Mature zygotic embryos of *Melia azedarach* (L.) were cultured on six culture media, all supplemented with 30 g/L sucrose and different PGRs combinations as follows:

1) MS/2 + 3 mg/L 1-naphthalene acetic acid (NAA) + 1 mg/L 6-benzylaminopurine (BAP) + 5 mg/L gibberellic acid (GA_3) (this PGR combination was suggested by Sharry *et al*. [16].
2) MS/2 + 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg/L BAP + 5 mg/L GA_3
3) MS/2 + 3 mg/L indole-3-butyric acid (IBA) + 1 mg/L BAP + 5 mg/L GA_3
4) MS/2 + 3 mg/L NAA + 1 mg/L Thidiazuron (TDZ) + 5 mg/L GA_3
5) MS/2 + 3 mg/L NAA + 1 mg/L zeatin + 5 mg/L GA_3
6) MS/2 + 3 mg/L NAA + 1 mg/L BAP

In sum, the first culture medium was that suggested by Sharry *et al.* [16]. In the other culture media, we replaced the auxin NAA with 2,4-D or IBA (culture media 2 and 3). Then we replaced the cytokinin BAP with TDZ or zeatin. In the last culture medium, we kept only the auxin-cytokinin combination without GA_3.

After one month of culture, all explants were transferred to the expression medium which consists of MS basal formulation supplemented with 10 mg/L BAP, 40 mg/L adenine, 161.1 mg/L putrescine and 60 g/L glucose.

Somatic embryo germination and development

After one month of culture, the regenerated somatic embryos were transferred to the germination and conversion medium which consists of MS medium supplemented with 0.2 mg/L BAP, 0.01 mg/L GA_3, 161.1 mg/L putrescine and 30 g/L sucrose.

Data collection and statistical analysis

Explants were placed in petri dishes. Each petri dish comprises 10 explants, which was considered as one replicate, and for each treatment, we used 10 replicates (i.e. the total number of explants per treatment was 100). At the end of the callus induction phase, the callogenesis rate of explants was calculated depending on each PGR combination. After one month in the expression medium, the percentage of calli/explants forming somatic embryos was recorded. After one month in the germination and conversion medium, the percentage of successfully germinated and converted somatic embryos into plantlets was calculated. Besides, callus morphology and somatic embryogenesis pattern (direct or indirect) were also monitored.

All experiments were conducted in a completely randomized design. Data were analyzed using ANOVA and the means were separated using the Student-Newman-Keuls test at 5% significance level. Before analysis, percentage data were arcsine transformed. Data were analyzed using SPSS v.16 for windows.

Results and discussion

Callus induction and morphology

In the present investigation, we attempted to develop a regeneration system through somatic embryogenesis for *Melia azedarach*. In fact, despite the high importance of this plant species, few studies were reported regarding its regeneration through *in vitro* culture. Mroginski and Rey [24], Thakur *et al*. [18] and Ahmad *et al.* [2] reported micropropagation of this plant species from axillary buds and nodal explants. Besides, adventitious organogenesis was also reported from leaflets of *in vitro* growing plants, apical meristem of *in vitro* regenerated shoots and leaf segments of adult trees [19, 20, 22]. Regarding somatic embryogenesis of *Melia azedarach*, it was reported from cotyledon explants and immature zygotic embryos [5, 16, 17, 21].

In this study, we evaluated the effects of six different PGR combinations on callus induction from mature zygotic

embryos of a *Melia azedarach* L. genotype growing in Morocco. Callus formation started after 10 days of culture in all the evaluated culture media. However, the callus induction rate and morphology varied depending on the PGR combination of the induction medium.

The callogenesis rate varied significantly, from 7 to 100%, depending on the PGR-combination of the induction medium (Table 1). The highest callogenesis rate was observed on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L zeatin and 5 mg/L GA_3. This was followed by the medium containing 3 mg/L IBA, 1 mg/L BAP and 5 mg/L GA_3, which showed a callus induction rate of 88%. The lowest callus induction rate (7%) was observed on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L TDZ and 5 mg/L GA_3.

Statistical analyses showed a significant difference between MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L TDZ and 5 mg/L GA_3 (7% callogenesis rate), and all the other culture media, which showed a callus induction rate ranging from 73 to 100%.

Table 1: Callus induction and somatic embryogenesis of Melia azedarach

Culture medium	Callus induction (%)	Callus morphology	Somatic embryogenesis expression (%)	Somatic embryogenesis pathway
MS/2 + 3 mg/L NAA + 1 mg/L BAP + 5 mg/L GA_3	81.0 a	White and friable	29.0 a	indirect
MS/2 + 3 mg/L 2,4-D + 1 mg/L BAP + 5 mg/L GA_3	73.0 a	Light brown with hydrated surface	-	-
MS/2 + 3 mg/L IBA + 1 mg/L BAP + 5 mg/L GA_3	88.0 a	White and friable	5.0 b	indirect
MS/2 + 3 mg/L NAA + 1 mg/L TDZ + 5 mg/L GA_3	7.0 b	Brown and compact	-	-
MS/2 + 3 mg/L NAA + 1 mg/L zeatin + 5 mg/L GA_3	100 a	White and friable	5.0 b	direct
MS/2 + 3 mg/L NAA + 1 mg/L BAP	73.0 a	Cream-white to yellow and friable	-	-

Data in the same column followed by the same letter are not different at the 5% significance level of the SNK test.

Regarding callus morphology, it varied depending on the induction medium. In MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L BAP and 5 mg/L GA_3, and that supplemented with 3 mg/L IBA, 1 mg/L BAP and 5 mg/L GA_3, we observed explant elongation along with callus development, which covers the whole explant surface. In some explants, we observed also swelling followed by callogenesis. All calli developed on these induction media were white and friable (Fig. 1a).

In MS/2 medium supplemented with 3 mg/L 2,4-D, 1 mg/L BAP and 5 mg/L GA_3, the initial explant has been completely transformed into a bulky cell mass. All the calli developed on this medium have a hydrated surface and a light brown color (Fig. 1b).

In MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L TDZ and 5 mg/L GA_3, the majority of the explants placed in culture did not show callogenesis. On the other hand, the very few explants that showed reaction to culture medium formed brown and compact calli that started from the radicle before covering the whole explant (Fig. 1c).

In MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L zeatin and 5 mg/L GA_3, the explants showed a winding-type morphology with a few amount of callus (Fig. 1d). All the calli were white and friable. These calli had the same morphological aspect as those developed on the medium supplemented with 3 mg/L NAA, 1 mg/L BAP and 5 mg/L GA_3, and that supplemented with 3 mg/L IBA, 1 mg/L BAP and 5 mg/L GA_3.

In the GA_3-free medium, the amount of callus was more important than that observed in all the other induction media, and all calli were friable with a cream-white to yellow color. In addition, in this culture medium, it seemed that the explant kept growing inside the formed calli.

The effects of PGRs on callus induction was reported in many plant species. For example, in *Phoenix dactylifera* L. [13] and in *Olea europaea* L. [12]. Besides, the morphology of calli also varied among plant species. Indeed, in some plant species such as olive, embryogenic calli have a brown color and were compact [12]. In date palm, embryogenic calli were white and friable [13]. In the present study, it was found that the explants of *Melia azedarach* may show different callus morphologies depending on the PGR combination of the induction medium.

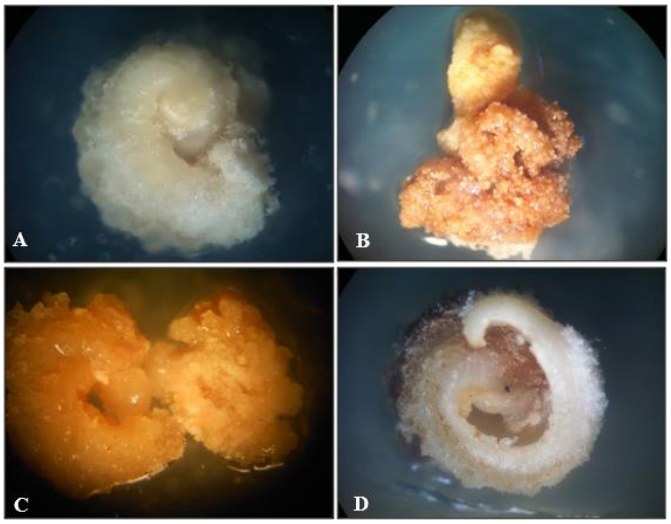

Fig 1: Different callus morphology observed depending on the induction medium. a Callus morphology on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L BAP and 5 mg/L GA_3. b Callus morphology on MS/2 medium supplemented with 3 mg/L 2,4-D, 1 mg/L BAP and 5 mg/L GA_3. c Callus morphology on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L TDZ and 5 mg/L GA_3. d Callus morphology on MS/2 medium supplemented with 3 mg/L NAA, 1 mg/L zeatin and 5 mg/L GA_3.

Somatic embryogenesis expression

After transferring the developed calli to the expression medium, different morphological aspects were observed depending on the induction medium. The cultures obtained from the medium containing the combination of NAA, BAP and GA_3 showed a change in their color. In fact, in the majority of cases, the calli became brown, especially at the contact area with the culture medium. After around 20 days of culture on the expression medium, somatic embryos started to arise from calli. Some of the calli obtained from the medium supplemented with IBA, BAP and GA_3 kept their white color while some others became slightly brown after their transfer to the expression medium. Here again, after around 20 days of culture, we noted the formation of somatic embryos (Fig. 1b). Vila *et al.* [21] reported direct somatic embryo induction within 10 days of culture from immature zygotic. This may highlight the effect of the explant age on somatic embryogenesis. In

fact, juvenile explants are known to be more responsive to *in vitro* manipulations than adult explants and thus the use of juvenile explants has been widely suggested to induce somatic embryogenesis [11].

Calli obtained from the medium containing 2,4-D, BAP and GA_3 showed an increase in their size and become more friable. However, no somatic embryo was observed. Regarding calli obtained on the medium containing NAA, TDZ and GA_3, no morphological change was noticed after their transfer to the expression medium. The calli developed on MS/2 medium containing only NAA and BAP showed a change in their color after transferring them in the expression medium and become brown. However, no somatic embryo was observed. On the other hand, the explants cultured on MS/2 medium containing NAA, zeatin and GA_3, showed somatic embryo formation. In this case, somatic embryos arose directly from the explant surface (Fig. 2b). Vila *et al*. [21] also reported direct somatic embryogenesis in *Melia azedarach*, but from immature somatic embryos. The PGRs used by these authors to induce direct somatic embryogenesis on immature zygotic embryos were TDZ (0.45-13.62µM) and 2,4-D (0.45 µM). In our case, the use of 2,4-D and TDZ did not induce somatic embryogenesis on mature zygotic embryos, which highlights the combined effects of the explant age and PGRs on somatic embryogenesis of *Melia azedarach*.

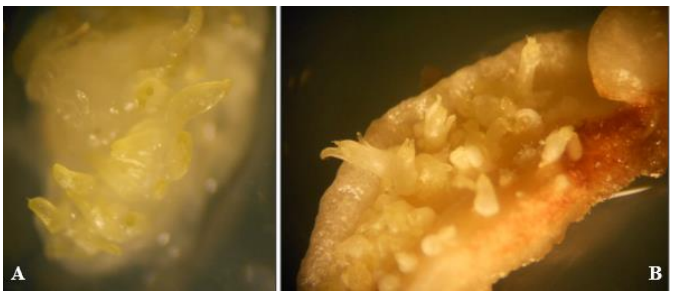

Fig 2: Somatic embryo expression. a Indirect somatic embryogenesis. b Direct somatic embryogenesis.

The highest rate of somatic embryogenesis was 29% on the medium containing the combination of NAA, BAP and GA_3, with a significant difference with all the other media (Table 1). This PGR combination was also suggested by Sharry *et al*. [16].

The effects of PGRs on somatic embryogenesis was observed in many plant species. In fact, the added PGRs interact with the endogenous plant hormones, which results in cell division and morphogenesis [6, 7, 8, 9]. However, the morphogenesis observed depends strongly on the genotype, explant and the PGR types and concentrations used. According to Vila *et al*. [23], in *Melia azedarach*, PGRs affect also somatic embryo morphology. In fact, somatic embryos produced from explants cultured on media containing BAP were similar to the zygotic ones, while those obtained on media containing 2,4-D and TDZ were morphologically different from zygotic embryos.

In the present investigation, the highest somatic embryogenesis rate was observed when the induction medium was supplemented with NAA, BAP and GA_3. This PGR combination appeared to be the most appropriate for *Melia azedarach* explants and thus could be recommended for regeneration through somatic embryogenesis in this plant species. Besides, many other factors were reported to influence somatic embryogenesis in *Melia azedarach.* Indeed, Vila *et al*. [23] indicated that explant stage, carbon source, gelling agent, light conditions and induction period all influence the somatic embryogenesis process of this plant species.

Somatic embryo germination and conversion into complete plantlets

After transferring somatic embryos to the germination medium (Fig. 3a), a germination rate of 51.28% was observed (Fig. 3b, c). Sharry *et al*. [16] reported successful germination of somatic embryos of *Melia azedarach* on different germination media, with and without PGRs, with up to 100% germination rate. The difference in the germination rate between our study and that of Sharry *et al*. [16] could be explained by the genotype effect. In fact, in both studies, the same PGR combination was used for embryo germination. Our findings showed that some somatic embryos were well individualized and gave larger shoots with numerous leaves, but did not show root formation. The embryos that successfully germinated (showed shoot and root formation) were all converted into complete plantlets and showed normal growth and development (Fig. 3d).

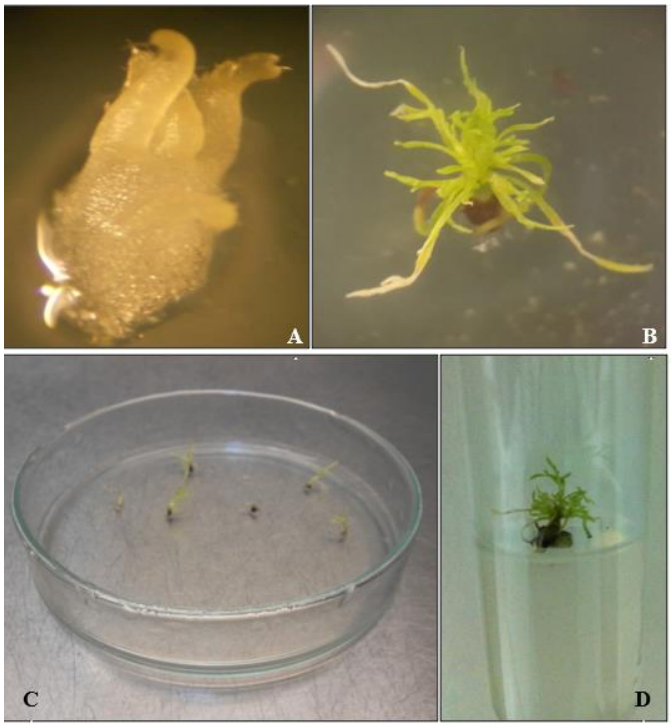

Fig 3: Somatic embryo germination and conversion into complete plantlet. a Somatic embryo placed on the germination medium. b and c Somatic embryo germination. d Somatic embryo conversion into plantlet.

Conclusions

We developed an efficient regeneration system through somatic embryogenesis for *Melia azedarach*. Callus induction was performed on MS/2 supplemented with 3 mg/L NAA, 1 mg/L BAP, 5 mg/L GA_3 and 30 g/L sucrose. Somatic embryos were formed from calli after transferring them on MS medium containing 10 mg/L BAP, 40 mg/L adenine, 161.1 mg/L putrescine and 60 g/L glucose while somatic embryo conversion into plantlets was carried out on MS medium supplemented with 0.2 mg/L BAP, 0.01 mg/L GA_3, 161.1 mg/L putrescine and 30 g/L sucrose. Our findings showed that somatic embryogenesis can be achieved either directly or indirectly from mature zygotic embryos of *Melia azedarach.* The results of our study will be beneficial for

further biotechnological investigations and applications for *Melia azedarach*.

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