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An Effective Protocol for the Production of Primary and Secondary Somatic Embryos in Cauliflower (*Brassica Oleraceae Var. Botrytis*)

Magda Al Shamari[1], Hail Z Rihan[2]* and Michael P Fuller[2]

[1]*Department of Horticulture Office, Ministry of Agriculture, Iraq*

[2]*School of Biological and Marine Sciences, Plymouth University, UK*

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***Corresponding author:** Hail Z Rihan, School of Biological and Marine Sciences, Faculty of Science and Engineering, Plymouth University, Plymouth, Devon PL4 8AA, UK, Tel: 00447513724273; Email: hail.rihan@plymouth.ac.uk

Abstract

An *in vitro* regeneration protocol based on the use of root-derived embryogenic callus tissue was established. Three different sizes of explants were tested in order to determine the optimal size of explants for somatic embryo production. The best formation of somatic embryos was on explant size 600-1000µm achieved using 90sec duration of blending. Kinetin played an important role in somatic embryo induction, development and maturation. Embryo maturation occurred on the medium with the lowest concentration of kinetin (0.5mg L^{-1}).

Normal secondary somatic embryos (SSEs) were induced directly on hypocotyls of primary somatic when cultured on semi solid MS basal medium supplemented with 30g L^{-1} sucrose in the absence of growth regulators. SSEs completed full development in the same medium. Cyclic secondary somatic embryogenesis was a good source of embryogenic material, which can be used for increasing the efficiency of plantlet regeneration in cauliflower. After 60 days of culture, it was demonstrated that using activated charcoal in the culture medium had no significant effect on SSEs yield and moreover, it had negative impacts on the morphological shape of embryos as most of SSEs were abnormal with split collar cotyledons and were smaller in size. The effective protocol for somatic embryo production designed in this study could have important applications in the field of cauliflower micropropagation and breeding systems.

Keywords: Embryogenic callus tissue; Kinetin; Somatic embryogenesis; Activated charcoal; Primary somatic embryos; Secondary somatic embryos; Abnormal somatic embryos; Cauliflower

Abbreviations: AC: Activated Charcoal; RDECT: Root-derived Embryogenic Callus Tissue

Introduction

Somatic embryogenesis is a multi-step *in-vitro* regeneration process, which starts with the formation of pro-embryogenic masses followed by somatic embryo formation, maturation, and plant proliferation [1]. The use of *in-vitro* somatic embryogenesis is preferred over other *in-vitro* developmental processes such as organogenesis or auxiliary bud propagation, since it can be used for micropropagation or genetic modification Ogita et al. [2] and for rapid proliferation of plants [3,4]. All plant species can probably achieve somatic embryogenesis when the appropriate explant, culture media and environmental conditions are optimised [5]. The efficiency of *in vitro* proliferation can be determined by explant size Gugsa & Kumlehn [6] and uniform culture conditions, which can be provided by the use of liquid culture systems. The change of medium is easier in the liquid culture production where bigger vessels can be used Sumaryono et al. [7] and the medium can easily be decanted. All of the surfaces of the explants are immersed using liquid medium and therefore nutrient adsorption can occur at all parts of the explants [8].

In most species, auxin and cytokinin are applied as the main plant growth regulators, which induce and allow the development of somatic embryos [9]. Plant growth regulators (PGRs) play an important role at the induction phase of somatic embryogenesis Pacheco et al. [10] and generally, somatic embryogenesis can be promoted by auxin alone George [11] or in combination with cytokinins [12]. Cytokinins can play a role in somatic embryogenesis by promoting cell division of pre-embryogenically determined cells [13].

Secondary embryogenesis, also termed recurrent, repetitive, accessory or proliferative embryogenesis occurs when primary somatic embryos cannot develop to plantlets. Second generation embryos can develop from cotyledons, hypocotyls or roots of

primary embryos [14-21]. In this phenomenon a new somatic embryo can be created from existing somatic embryos [22]. The secondary somatic embryogenesis system can be used in plant breeding Shu & Loh [23] since repeated cycles of secondary embryogenesis can maintain the "embryogenicity" for prolonged periods of time Raemakers et al. [24] without apparent diminution of embryogenic capacity [23]. It can also be useful for the production of artificial seed [25,26] since unlimited numbers of somatic embryos can be provided by the repetitive culturing [27]. Secondary embryos have been reported in several brassica species such as *Brassica rapa* (Chinese cabbage) Choi et al. [28] *Brassica nigra* (Black Mustard) Gupta et al. [29] and *Brassica napus* (Rapeseed) Shu & Loh [23], Burbulis et al. [30], Koh & Loh [31] but there is little literature on secondary embryogenesis in vegetable brassicas especially in cauliflower (*Brassica oleracea var. botrytis*).

In a wide range of plant species, the addition of Activated Charcoal (AC) in culture media generally promotes growth, somatic embryogenesis and organogenesis [32-35]. The applicability of AC in the culture medium comes from its general ability for the adsorption of inhibitory substances Thomas [36] and particularly phenyl acetic acid, benzoic acid derivatives and other colourless toxic compounds produced during embryogenesis [37,38]. In tissue culture, AC often promotes morphogenesis and this might be mainly due to its ability to decrease toxic metabolic brown exudate accumulation and phenolic exudation common in plant tissue culture [36]. In vitro, there are both positive and negative effects of AC depending on different factors especially its concentration, the plant species cultured and the type of explant tissue used [33,39,40].

The current study aimed to optimise an effective protocol for primary and secondary somatic embryo production system in cauliflower. This included the determination of the best explant size class that should be used to produce efficient somatic embryo production in cauliflower and the investigation of the influence of kinetin in this process. To our knowledge, there have been no studies reporting the effect of AC on secondary somatic embryos formation in cauliflower and cauliflower has been considered to be recalcitrant to somatic embryogenesis. The work reported here focussed on assessing AC effects on quantitative and qualitative *in vitro* yield of SSEs from primary somatic embryos.

Materials and Methods

Cauliflower somatic embryo production system and the effect of different sizes of root-derived embryogenic callus

Continuous immersion in agitated liquid media technique (CI ALMT) was used to induce somatic embryo formation on the explants of root-derived embryogenic callus tissue (RDECT). Cauliflower cv. White Cloud, was used throughout this study. In this technique, the explants (Figure 1A) were homogenized using a commercial blender (Multi-mixer model no. 50376) (Figure 1B). Blending was made with 50mL of MS basal medium [41]. The explants derived from the blending were then classified into three size classes (300-600, 600-1000 and 1000-2000µm) using commercial sieves (Endecotts Ltd., London) (Figure 1C). After sieving, a consistent volume of explants (74µL) was placed in culture pots containing 30mL of somatic induction medium (SIM) which consisted of 0.05mg L^{-1} IAA, 0.5mg L^{-1} Kinetin and 2% sucrose. Five pots of each treatment were placed randomly on an orbital shaker at 150 rpm and supplemented with 16h light (spectral photo fluence 40µmol m^{-2} s^{-2}) supplied with cool white fluorescent tubes. After 40 days culture, somatic embryos on each explant were observed under a low power light microscope (EMZ-8TR) fitted with a camera (Infinity 2) and the number of embryos counted and developmentally classified.

Figure 1: cauliflower somatic embryo production system.

Optimization of blending duration

Six blending durations (15, 30, 45, 60, 75 and 90sec) were conducted using 3g of RDECT for each blending treatment. The total amount of explants was evaluated as fresh weight and volume of explants produced from each sieving size class. Two size classes, 600-1000µm and 1000-2000µm, of homogenised RDECT were used in this experiment.

Effects of cytokinin on somatic embryo production

Chopped RDECT of size 600-1000µm were placed on SIM medium supplemented with various concentrations of kinetin (0.5, 1, 2mg L^{-1}) together with 0.5mg L^{-1} IAA and 20% sucrose to assess the effect of kinetin concentrations on somatic embryo induction, development and maturation. Each treatment consisted of five pots, each containing 30mL of culture medium

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with a constant volume (74µL) of explants. Callus cultures were grown and developed in this medium for 40 days. The development and maturation of embryos were observed and the number of embryos recorded. The embryogenesis rate was assessed as the number of explants that produced somatic embryo per volume unit used of explants (74µL).

Explant preparation for secondary somatic embryos production

A simplified procedure was developed to produce secondary somatic embryos (SSEs) from primary somatic embryos (PSEs) of cauliflower. Primary somatic embryos at the cotyledonary stage were produced from root-derived embryogenic callus tissue on somatic induction medium (SIM). The media consisted of MS supplemented by 0.05mg L^{-1} IAA (Indole-3-acetic acid), 0.5mg L^{-1} kinetin and 20% sucrose. Primary somatic embryos were isolated and collected to use as source material for the induction of SSEs.

Secondary somatic embryos production

To find the best medium for secondary somatic embryogenesis and embryo maturation, 25 embryos were used for each treatment, 5 mature PSEs were transferred into each pot (5 pots for each treatment) each containing 30mL of secondary somatic induction medium. Two types of induction medium were used, the first consisted of semi-solid MS medium free of growth regulators plus 30g L^{-1} sucrose and the second type was MS medium with activated charcoal at two concentrations (1 and 2g L^{-1}). The pots were kept (completely randomised) in a growth incubator at 22.5 °C under 16 photoperiod at a light intensity of 80µmol$m^{-2}s^{-1}$ PAR supplied by cool white fluorescent tubes and this experiment was repeated three times. Secondary somatic embryos were observed and classified (globular, torpedo, heart shaped, cotyledonary) under a low power light microscope (EMZ-8TR) fitted with a camera (Infinity 2). The number and quality of SSEs were recorded after 60 days of culture.

Statistical analysis

Results are presented as means with standard errors (S.E.). All data were subjected to analysis of variance (ANOVA) using Minitab software (version 15) and comparisons of means were made with least significant difference test (LSD) at the 95% level of probability.

Results

Effect of sieving size class on the formation of somatic embryos

The results revealed that the optimal formation for somatic embryos from RDECT explants was achieved from size class 600-1000µm ($P \leq 0.001$) with significant less produced at the higher and lower sieving size classes. The highest value for the somatic embryo number (30.3) was produced from explants size 600-1000µm while poor embryo formation was produced from explants size classes 300-600 and 1000-2000µm (Figure 2).

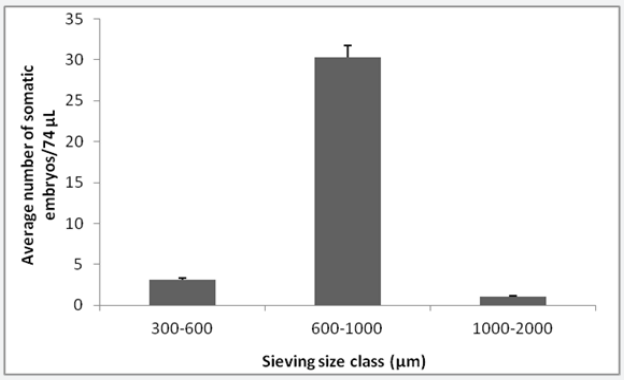

Figure 2: The effect of explant size class on the production of somatic embryos (LSD=5.7).

Effect of blending duration on the volume and weights of explants produced

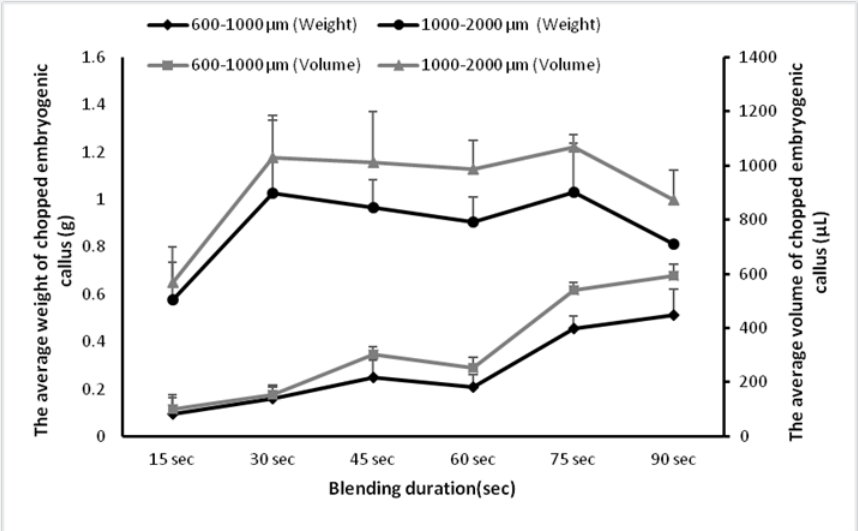

Figure 3: Effect of blinding duration on the volume and weights of explants produced (LSD for weight = 0.264 and for the volume= 265.059).

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The results showed that the use of 90 sec blending duration was found to be optimal as it produced the highest amount of explants in terms of fresh weight and volume at the desirable size class (600-1000µm). However, at the 1000-2000µm size class, the highest amount of explants produced was with the 75sec blending duration (P≤0.001) (Figure 3).

Effect of kinetin concentration on the formation of somatic embryos

The results indicated that the medium containing 0.5mg L^{-1} Kinetin and 0.05mg L^{-1} IAA produced the maximum total number of somatic embryos (30.9 embryo/explants) (P≤0.001) with the highest embryogenesis rate 60% (Figure 4). It was observed that the development of somatic embryos in this medium was also qualitatively better as the somatic embryos developed sequentially from globular to heart to torpedo and reached cotyledonary stage. While embryogenesis was observed on 57% of explants on the medium supplemented with 1mg L^{-1} Kin plus 0.05mg L^{-1} IAA, these stayed mostly at globular and heart stages with only some conversion to the other later stages of development. It was also noted that a high number of somatic embryos on the medium containing 2mg L^{-1} Kin plus 0.05mg L^{-1} IAA stayed at the globular stage despite an embryogenesis rate of 55% (Figure 5).

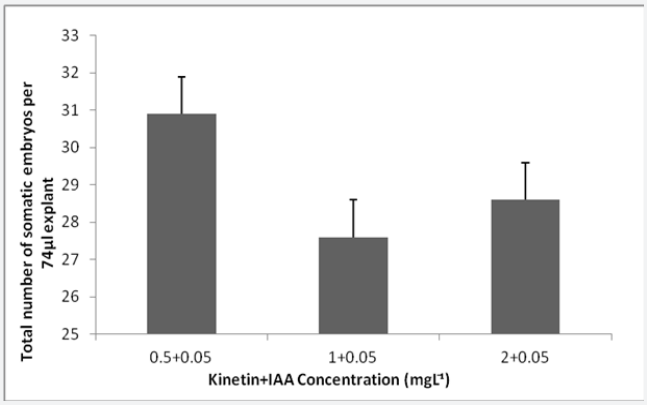

Figure 4: Effect of kinetin concentration on the formation of somatic embryos (LSD=8.99).

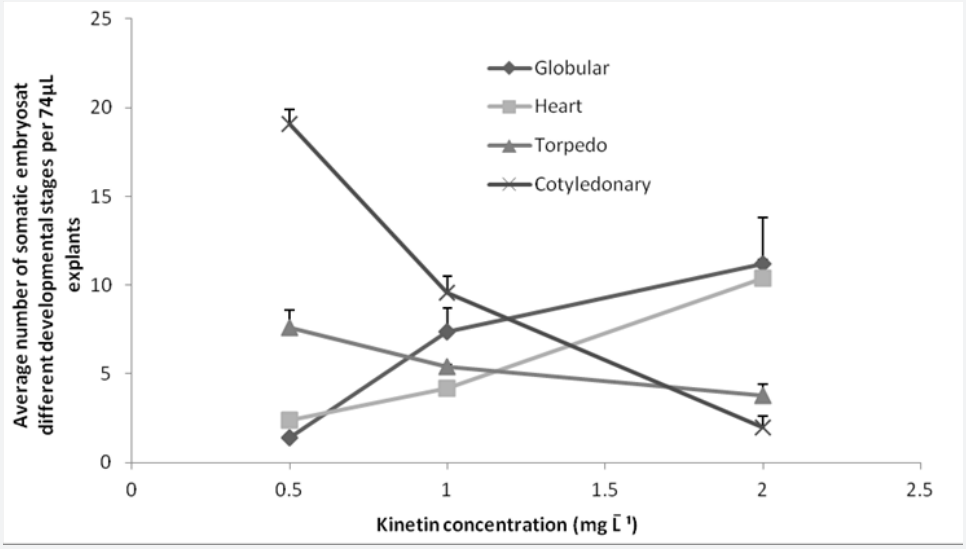

Figure 5: The effect of kinetin concentration on the development of somatic embryos (LSD for globular stage = 4.93, LSD for heart stage = 4.40, LSD for torpedo stage = 4.34 and LSD for cotyledonary stage = 5.75).

Effect of AC on SSEs production

The results revealed that the use of PSEs as explants led to the formation of secondary embryos on MS basal medium free of hormones supplemented either with or without activated charcoal (AC). SSEs were visible from the PSEs within 60 days of culture. It was observed that a small mass of tissue proliferated from the tissue corresponding to the hypocotyls of primary embryos, after that several SSEs emerged. Different developmental stages of SSEs were noticed. However, secondary embryos developed directly on hypocotyls of primary embryo (Figure 6A). Primary somatic embryos on MS basal medium (free of hormones and AC) exhibited the best induction for normal SSEs (embryos with two cotyledons) producing 9.2 embryo/explants (Tables 1 & 2) but there were no significant differences between treatments in terms of total number of SSEs (Tables 1 & 2).

Two abnormal morphological types of SSEs were observed. When AC was added to the media, embryos with split collar cotyledons (Figure 6B) were obtained on MS medium plus 1 and 2g L^{-1} AC while abnormal SSEs with four cotyledons were sometimes observed on plain MS induction medium. The size of secondary embryos that formed on MS medium without AC differed significantly (P≤0.001) and the highest average of embryo size (4.2mm) was achieved using AC free medium. However, the PSEs could bear smaller SSEs along their hypocotyl region when placed on medium with AC.

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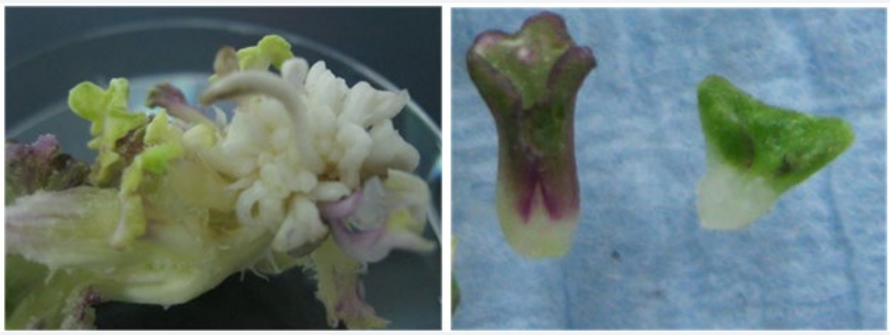

Figure 6: (SCC: split collar cotyledons, PSE: primary somatic embryos and SSEs: secondary somatic embryos).

Table 1: The effect of activated charcoal (AC) on normal and abnormal secondary somatic embryo (SSE) formation from hypocotyls of primary somatic embryos and the number of shoots produced per secondary somatic embryo.

AC Concentration Gl^{-1}	Total Number of Sses	Number of Normal Sses	Number of Abnormal Sses (4 Cotyledons)	Number of Abnormal Sses (Collar Cotyledons)	Number of Shoots	Embryo Size(Mm)
0	13.2a	9.2a	4.0a	0b	0b	4.2a
1	14.2a	0.8b	0b	13.4a	1.6ab	2.0b
2	13.8a	1.2b	0b	12.6a	2.4a	1.4b

Table 2: Least Significant Difference values.

	LSD
Total number of SSEs	6.66
Number of normal SSEs	3.34
Number of abnormal SSEs(4 cotyledons)	0.56
Number of abnormal SSEs(collar cotyledons)	5.37
Number of shoots	1.88
Embryo size	0.97

The results also showed that the MS medium supplemented with 1 and $2gL^{-1}$ AC had a positive effect on subsequent shoot formation as these media led to few shoots being formed (1.6 and 2.4 shoots) using 1 and $2gL^{-1}$ respectively and no shoots formed on AC free MS medium (Tables 1 & 2).

Discussion

Using the continuous immersion in agitated liquid media technique (CI ALMT), somatic embryos were readily produced from explants of root derived embryogenic callus tissue (RDECT). The direct and indirect regeneration of somatic embryos on explants depended largely on the size of the explants. This emphasises the need to empirically optimize the explant size [5]. Many histological studies on somatic embryogenesis induction have suggested that somatic embryogenesis starts with active division in embryogenic callus which then develops into an embryo-like structure that then advances through the familiar globular, heart, torpedo and cotyledonary developmental stages [42-46]. In the current study, although somatic embryos with all developmental stages were produced in callus cultures from all the explants size classes tested, significant numbers of somatic embryos was only achieved in cultures produced from explant size class 600-1000 µm with many less from both smaller and larger explant sizes. In sweet potato, it was similarly observed that the decrease in the size of cell aggregates can lead to a reduction in somatic embryos produced [47]. Shigeta & Sato [48] cultured horseradish embryogenic callus in MS liquid medium and after four weeks, somatic embryos were only obtained from a cell aggregate size of 1000 µm or less in diameter. Wannarat [49] obtained horseradish somatic embryos from specific sized cell aggregates ranging from 1000-3000 µm in diameter whilst Chen et al. [50] reported that the smaller pieces of embryogenic calli that were derived from leaflets of sexual bahiagrass which were less than or equal to 2000µm in diameter appeared uniform in size and hadabilities for regeneration. Jain et al. [51] mentioned that the development of date palm callus growth and improvement of synchronized somatic embryos can be achieved when fine chopping of embryogenic callus into small pieces <380µm was applied.

When using mechanical blending techniques, it is important to determine the best blending duration and here it was noted that the amount of cauliflower explants produced for a desirable size class differed with blending durations used. It was demonstrated that increasing blending duration to 90sec led to the production of a good amount of explants at size class 600-1000µm. The use of a blending technique has also been used effectively to produce suspension cultures from callus tissues of *Capsicum frutescens* Williams et al. [52] and to homogenize callus tissues [53-56].

Plant growth regulators can play essential roles in somatic embryogenesis induction Toonen & Devries [57] but the requirements of auxin and cytokinin and their levels have to be determined empirically for each plant species [58]. Cytokinins appear to have a role in the initial cell division phase of somatic embryogenesis Danin et al. [59] and the responsibility for the

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establishment and maintenance of embryo apical meristems can be achieved by cytokinins [7]. In *Brassica rapa*, the presence of growth regulators in the basal medium led to the development of somatic embryos [60]. Kinetin in combination with auxin, particularly IAA was used to produce somatic embryos in cauliflower Pareek & Chandra [61] and our findings showed that the growth and development of somatic embryos were influenced by Kinetin concentration. Globular embryos developed into heart and torpedo shaped embryos and converted into cotyledonary shapes faster on media containing low concentrations of Kinetin (0.5mg L^{-1}). Moreover, a high number of embryos on media containing 1 or 2mg L^{-1} Kinetin with 0.05mg L^{-1} IAA could not develop to the cotyledonary stage. This effect of high level of cytokinin partially or totally inhibiting the development of somatic embryo cotyledons has also been reported previously by Ammirato [62].

Secondary embryos are thought to arise directly from epidermal or sub epidermal cells of cotyledons or hypocotyls [63]. In the current study the appearance of secondary embryos was from the hypocotyl region of primary somatic embryos after 60 days of culture and this in accordance with Kumar & Shekhawat [64] who commented that prolonged culturing leads to the proliferation of secondary embryos. According to our results and others studies conducted on *Brassica nigra* Gupta et al. [29] and *Brassica napus* [30,65-67] report the production of secondary embryos using MS medium free of growth regulators. Gupta et al. [29] had concluded that six to seven secondary embryos were obtained from *Brassica nigra* protoplast-derived embryos. Also in Chinese cabbage, a few secondary embryos were formed on the surface of somatic embryos [28]. Habituated cultures can be defined as the cultures that have the ability to proliferate in culture medium in the absence of exogenous growth regulators [68]. In some species, the maturation of embryos does not require additional culture steps Raemakers et al. [24] and this appears to be the case with cauliflower, where the secondary somatic embryos developed and matured on the same induction medium.

Somatic embryos can be classified into normal or aberrant (morphologically abnormal in size and shape or lacking distinct stages) [69]. In the current work it was demonstrated that the yield of normal SSEs did not improve significantly when AC was added to the medium. A similar effect was previously reported by Aderkas et al. [70] with somatic embryo production of hybrid larch. The development of abnormal somatic embryos such as the split-collar cotyledon somatic embryos can be obtained when changes in auxin distribution or activity occur during the transition from globular to heart stage. During transition, the separation of the emerging cotyledonary primordial ring into two parts occurs, and by this process bilateral symmetry is achieved around the apical-basal axis. Polar transport for auxin can play an essential role in this process. Polar transport leads to the removal of auxin from the area between the two emerging cotyledon primordia as well as providing a continuous transport for auxin which is a prerequisite until the separation of the cotyledon primordia can be observed morphologically. The split-collar cotyledons occur when the partial separation of the cotyledons is obtained [71]. In the current study the appearance of split collar cotyledon embryos was on media containing AC and it has previously been reported that AC can adsorb auxin from culture media Constantin et al. [72] including perhaps excessive quantities of both exogenously supplied and tissue-produced auxins [73]. This suggests that, the absorption of auxin that is released by the embryos to the medium during embryo development may interfere with morphology and germination of embryos [74]. We suggest that AC may have an effect on internal auxin concentration and its activity resulting in the appearance of abnormal SSEs with the split-collar cotyledons shape. The other abnormal shape that appeared in the current study on MS medium was embryos with four cotyledons. Abnormal somatic embryos with more than two cotyledons have been observed before [75,76] and has been described previously in cauliflower by Leroy et al. 2000. This might be due to prolonged time in culture that can cause accumulation of mutations (somaclonal variations), which can lead to morphological abnormalities such as multiplex apical formation, pluri-cotyledonary structures and fused cotyledons [77]. According to our findings, the SSEs that formed on media enriched with AC appeared morphologically smaller in size (1.4mm) compared with those originating on MS medium devoid of AC (4.2mm). In contrast Pintos et al. [78] commented that a significant increase in a relative size of cork oak somatic embryos can be enhanced using AC in the medium and embryo size increased to reach 1.3cm in length. A compromise between the number and quality of SEs developed and the use of AC has been always complex and species specific and some authors maintain that the use of AC in embryogenesis remains questionable [79].

Another observation in the current study was the development of some shoots by direct organogenesis from hypocotyls of primary somatic embryos in the presence of AC. Previously, it was reported that a stimulative effect on the growth and organogenesis of different plants is achieved when AC is added to the culture medium [80]. Similarly, Nayanakantha et al. [81] found that the addition of AC to MS medium led to shoot induction and multiplication of *Aloe vera*.

Conclusion

A reliable method was developed to produce somatic embryos from RDECT of cauliflower and the optimization of culture conditions for induction and development of somatic embryos was determined. It was clear that the use of CI ALMT with root derived explants was a very efficient tool for the high proliferation of somatic embryos of cauliflower. The explant size had an overriding effect in terms of the number of somatic embryos produced and the exogenous Kinetin level affected both the development and maturation of the somatic embryos.

It was also concluded that primary somatic embryos can be successfully used to produce secondary somatic embryos in cauliflower. It was found that the use of MS medium devoid of

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growth regulators could be very efficient for the good proliferation of normal secondary somatic embryos of cauliflower. The addition of activated charcoal to the medium did not enhance the production of SSEs and led to some malformations.

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