CHAPTER 4

PLANTLET REGENERATION OF *PROTEA CYNAROIDES* THROUGH DIRECT SOMATIC EMBRYOGENESIS AND MULTIPLE SHOOT DEVELOPMENT

4.1 Abstract

Little research has been done on the *in vitro* propagation of *Protea cynaroides*. Somatic embryogenesis is often used to propagate a number of plant species and can potentially be used to rapidly propagate *P. cynaroides*. From the results of this study, somatic embryos formed directly on both *P. cynaroides* mature zygotic embryos and excised cotyledons cultured on MS medium without growth regulators. The addition of growth regulators such as NAA (1; 2.5 and 5 mg l^{-1}) and 2,4-D (1; 2.5 and 5 mg l^{-1}), in combination with TDZ (0.2 mg l^{-1}), BAP (0.2 mg l^{-1}) or kinetin (0.2 mg l^{-1}) suppressed the formation of somatic embryos. After eight weeks in culture, formation of somatic embryos was observed. Zygotic explants formed the most embryos when cultured in a 12-hour photoperiod in comparison to explants cultured in the dark. Up to 83% of these embryos germinated after transferal to the germination medium containing 0.1 mg l^{-1} GA_3. Significantly fewer embryos germinated in MS medium with no growth regulators, or supplemented with higher concentrations of GA_3, while low germination percentages were also observed in MS media containing casein hydrolysate and coconut water. The germination of normal embryos was observed only in medium containing either no growth regulators, 0.1 mg l^{-1} GA_3 or 0.5 mg l^{-1} GA_3. All embryos that germinated in high concentrations of GA_3 were malformed.

4.2 Introduction

Plant regeneration *via* somatic embryogenesis has the potential to produce a large number of plantlets in a relatively short period of time. Although numerous plant species are reportedly capable of forming somatic embryos, very few reports of somatic embryogenesis have been documented for members of the Proteaceae family. In the *Protea* genus only *Protea repens* have been reported to form somatic embryos

(Rugge, 1995). Van Staden, Choveaux, Gilliland, McDonald and Davey (1981) were able to induce callus and proteoid rootlet formation in *Protea neriifolia*, but attempts to initiate shoot and root development were unsuccessful. In another commercially-important Proteaceae species, *Serruria florida*, explants were induced to form somatic embryos (Rugge, van der Merwe, Jacobs and Theron, 1989).

The slow growths of established explants, as well as their inability to form roots, are stumbling blocks encountered during micropropagation of *P. cynaroides* (Wu and du Toit, 2004). This was shown by Ben-Jaacov and Jacobs (1986) and Wu and du Toit (2004), where *P. cynaroides* explants were established successfully *in vitro* by inducing axillary buds to sprout. However, *in vitro* rooting of those explants was unsuccessful. In addition, phenolic oxidation of the explants has been reported to be a problem, which resulted in their death. Thus, somatic embryogenesis can potentially be used as an alternative *in vitro* propagation method to produce *P. cynaroides* plantlets.

Histological studies have been used to investigate cell, tissue and organ development in somatic embryos (Stamp, 1987; Samaj, Bobak and Erdelsky, 1990; Puigderrajols, Celestino, Suils, Toribio and Molinas, 2000). Histological studies are also conducted to investigate the presence of starch grains in plant cells, which are revealed by various types of stains applied to the plant tissue. It is often reported that starch serves as an energy source for cell division and tissue formation in somatic embryos (Samaj *et al*., 1990). Thus, the identification of starch grains in a certain region of an embryo is indicative of active tissue development.

The main objective of this study was to investigate and determine the ideal explant, optimum growth conditions and suitable culture medium for the induction, development and germination of somatic embryos. This in turn would allow the development of a protocol for somatic embryogenesis of *P. cynaroides* from which plantlets could be produced rapidly.

4.3 Materials and methods

4.3.1 Plant materials

Excised, mature zygotic embryos, cotyledons from *in vitro*-germinated seedlings and immature unfertilized ovules were used as source material for the induction of somatic embryos. The methods of excision and sterilization of the mature zygotic embryos are described in Chapter 2. The cotyledons were obtained from newly *in vitro*-germinated seedlings and cut into similar sizes (5 mm x 5mm). All the cotyledons were placed on their adaxial side on the growth medium. The immature unfertilized ovules were collected from 30-day old inflorescences of 5-year old established *P. cynaroides* motherplants grown in a field situated near Cullinan (25º40'32S; 28º31'20E; Altitude 1482 metres) in the Highveld region (summer rainfall) of South Africa. After the ovary was carefully opened with a sterile needle, the immature unfertilized (haploid) ovule was removed and placed onto the culture medium (Figure 4.8A).

4.3.2 Culture media and growth conditions

For the induction of somatic embryos, forty explants were used in each medium treatment. Full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Appendix A) and 3% (w/v) sucrose and Gelrite® (3 g l^{-1}) was used as the basal medium. The pH of all the media was adjusted to 5.7 before autoclaving. 2,4-Dichlorophenoxyacetic acid (2,4-D) (1, 2.5 and 5 mg l^{-1}) and 1-naphthalene acetic acid (NAA) (1, 2.5 and 5 mg l^{-1}) were either added singly or in combination with the following: thidiazuron (TDZ) (0.2 mg l^{-1}), 6-benzylaminopurine (BAP) (0.2 mg l^{-1}), or kinetin (0.2 mg l^{-1}). Other media supplements included coconut milk (5% and 10%), and casein hydrolysate (100 mg l^{-1} and 200 mg l^{-1}), which were added singly into the basal medium containing no growth regulators. In addition, full-strength basal MS medium with no growth regulators was also used as induction medium.

In the germination study, thirty embryos in the heart-shaped stage, which formed in the induction stage, were carefully removed from the source explants and transferred onto sterilized filter paper (Whatman No. 1) lining 9-mm Petri dishes containing

germination medium. Half-strength MS medium was used with the following growth regulators added singly: GA_3 (0.1, 0.5, 1, 2.5, 5, 10, 20 mg l^{-1}), casein hydrolysate (100, 200 mg l^{-1}) and coconut water (50, 100 mg l^{-1}). Half-strength basal MS medium with no growth regulators was also used as a germination medium.

For the induction of somatic embryos, the explants were either cultured under a 12-hour photoperiod or in total darkness. Illumination was supplied by cool, white fluorescence tubes providing 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). For germination, somatic embryos were grown in the dark only. The temperature was adjusted to 25±2ºC for the induction stage, while 21±2/12±2ºC was used in the germination stage since this alternating temperature regime has shown to improve germination of zygotic embryos in the previous study (See Chapter 2).

4.3.3 Histology

Anatomical investigations were performed on the somatic embryos to observe the presence of starch grains and study the histodifferentiation of somatic embryos through the various stages. The methods used in the preparation of microscope slides were adapted from O'Brien and McCully (1981). Somatic embryos in the globular, heart and cotyledonary stages were first fixed in formalin: acetic acid: 50% ethanol (1: 1: 18), and then dehydrated in an ethanol: distilled-water series (30:70; 50:50; 70:30; 100:0). Finally, the sample was embedded in paraffin wax (melting point 60ºC). Samples were cut in a semi-thin rotary microtome (Reichert-Jung 2040®) at a thickness of 7 µm. Sections were attached to glass slides and stained with safranin and fast-green, which are used to reveal the presence of starch grains (Peacock, 1935; Plata and Vieitez, 1990). A Wild Leitz GMBH® (Model 020-057.010) light microscope was used to observe the slides at 50x and 500x magnification.

4.3.4 Statistical analysis

In the somatic embryo induction, the percentage of explants that formed embryos, and the number of embryos formed per explant was recorded. For the germination stage, the total number of embryos that germinated per treatment, as well as the number of normal-germinated embryos were recorded. A completely randomized design was

applied Where appropriate, Chi-Square analysis and Tukey's Studentised range test were used to compare treatment means. All statistical analyses were done in the SAS program (SAS Institute Inc, 1996). ANOVA is shown in Table C3, Appendix C.

4.4 Results

4.4.1 Induction of somatic embryos on zygotic embryos

4.4.1.1 MS medium without growth regulators

Six weeks after culturing the explants on the induction medium, the first somatic embryos began to appear on the zygotic embryos cultured on full-strength basal MS medium with no growth regulators (Figure 4.1). However, the majority of somatic embryos were formed after eight weeks in culture. All of these somatic embryos differentiated directly, without an intermediate callus phase. Somatic embryos formed on edges (Figure 4.1A), the abaxial side (Figure 4.1B) and the adaxial side (Figure 4.1C) of the zygotic embryo. Somatic embryos formed on the basal MS medium under both light (12-hour photoperiod) and dark conditions, although under light conditions, significantly more ($P \leq 0.001$) explants formed embryos (70%), compared to 37.5% of explants forming embryos in the dark (Table 4.1). Moreover, significantly more embryos were formed per explant under the light treatment (8.7 embryos) than in the dark (5.5 embryos) (Table 4.1). The somatic embryos that formed on these basal MS media were able to develop from the globular through to the heart stages, with some embryos developing further into the torpedo-shape stage (Figure 4.1D). In addition, a low number of embryos germinated when kept on MS medium with no growth regulators.

4.4.1.2 MS medium supplemented with growth regulators and other additives

After 8 weeks, somatic embryos also appeared on zygotic embryos cultured in the MS media containing coconut water or casein hydrolysate. However, the number of zygotic explants that formed embryos was less than 10%, and these results were considered insignificant. In addition, all the media containing various concentrations

of 2,4-D or NAA, singly or in combination with TDZ, BAP or kinetin, cultured under light or dark treatments did not induce formation of any somatic embryos, nor were there signs of embryogenic callus formation. One exception to this trend was found on MS medium containing 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP in the dark, where explants showed some form of callus growth, however, no somatic embryos were produced. In general, zygotic embryos cultured on MS medium containing growth regulators or other additives formed fluffy, watery-looking callus (Figure 4.3A). Attempts to induce embryos on these calluses were unsuccessful. Eventually, these calluses turned brown and died.

4.4.2 Induction of somatic embryos on cotyledon explants

4.4.2.1 MS medium without growth regulators

After 8 weeks in culture, somatic embryos also formed directly on cotyledonary explants grown on full-strength MS medium lacking growth regulators, under both light and dark conditions (Table 4.1; Figure 4.2). These embryos developed through the globular to the heart and torpedo stages (Figure 4.2C; Figure 4.2D). The percentage of cotyledonary explants that produced embryos was significantly less ($P \leq 0.001$) than those produced by the zygotic embryo explants cultured on the same medium treatment and light conditions (Table 4.1). Twenty-five percent and 22.5% of cotyledons formed embryos in light and dark conditions, respectively. In addition, the number of embryos formed per explant was significantly less than the amount formed on zygotic explants (Table 4.1).

4.4.2.2 MS medium supplemented with growth regulators and other additives

Similar to the zygotic explants, the direct formation of somatic embryos was suppressed by the addition of growth regulators to the culture medium. Only a few watery calluses formed scantily, and no embryos were induced on the excised cotyledons cultured on any MS medium supplemented with growth regulators. Figure 4.3B illustrates the effect of growth regulators such as 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP on the callusing of cotyledonary explants. These explants were covered in watery callus, and showed no ability to produce somatic embryos. Furthermore,

unlike the zygotic cultures, in light, no embryos were formed in the medium containing coconut milk or casein hydrolysate, while less than 10% of these explants formed embryos in the dark.

4.4.3 Germination of somatic embryos

Histodifferentiation of somatic embryos was rapid from the heart stage to the cotyledonary stage (Figure 4.4). Germination of somatic embryos was observed in all the germination media containing different concentrations of the various growth regulators. However, the germination percentage varied widely from 10% to 83% (Table 4.2). Although germination of the embryos was achieved in the medium lacking growth regulators, germination was favoured in medium supplemented with GA_3. After four weeks in culture, highest germination percentage was observed in the medium containing 0.1 mg l^{-1} GA_3 (83.3%), which was significantly higher ($P \leq 0.001$) than the medium with 0.5 mg l^{-1} GA_3 (60%) (Table 4.2). Significantly fewer embryos germinated in media supplemented with higher concentrations (1 - 20 mg l^{-1}) of GA_3, casein hydrolysate or coconut water. Furthermore, 70% of the embryos that germinated in 0.1 mg l^{-1} GA_3 were normal, i.e. they consisted of two separate cotyledons, and a single radicle (Figure 4.5). Somatic embryos which germinated in the medium containing either no growth regulators or 0.5 mg l^{-1} GA_3 yielded only 55.6% and 50% normal germination, respectively, while in all the other media treatments, the majority of embryos that germinated were malformed. The malformed embryos either had single, multiple or fused cotyledons, or fused embryos (Figure 4.6).

Somatic embryos at globular, heart and cotyledonary stages are illustrated in Figure 4.7. Undifferentiated epidermal cells occur around the entire globular embryo (Figure 4.7A), while the development of the radicle and shoot poles is revealed by the presence of smaller differentiated cells at each polar end (Figure 4.7A). In the heart-shaped embryos, accumulation of starch grains occurs in the vicinity of the developing cotyledons, indicating that cell division and tissue growth is taking place (Figure 4.7B). In the cotyledonary-stage embryo, the vascular tissues are visible along the centre of the two cotyledons, which connect up toward the upper centre of the embryo (Figure 4.7C). Starch deposits are also abundant in cells in the immediate

vicinity of the vascular tissues, particularly on the left side of the somatic embryo (Figure 4.7C). Fewer cells containing starch were found in the lower part of the embryo, especially towards the radicle pole (Figure 4.7C). Figure 4.7D illustrates a close-up of starch granules that are stored in slightly elongated parenchyma cells in a region close to the vascular tissue where cell activity are taking place.

4.4.4 Immature unfertilized ovules

Use of full-strength MS medium in all media treatments led to the browning and death of most immature unfertilized ovules. Half-strength MS medium was then used in place of full-strength MS medium, while growth regulator concentrations remained the same. Results showed that continuous darkness is required for immature unfertilized ovules to grow. The majority of explants cultured in the dark showed significant growth, while a low percentage of ovules cultured in light responded positively (Table 4.3).

Ovule explants cultured in the dark expanded rapidly, and tissue growth was observed after 5 days (Figure 4.8B). This rapid growth of the ovules continued after 4 weeks in culture (Figure 4.8C), and was similar in appearance at all medium treatments supplemented with various concentrations of auxins and cytokinins. However, the rapid growth of ovules decreased considerably after 8 weeks in culture, which is shown by their similarity in size to when they were 4-weeks old (Figure 4.8D). No differences in the appearance of the ovules could be observed between the various medium treatments after 8 weeks. Significant differences were, however, observed in the amount of ovules that showed tissue growth and expansion (Table 4.3). A high percentage of ovules (100%) cultured on MS medium without growth regulators showed tissue expansion and growth, while significantly fewer ovules showed growth on media supplemented with low concentrations (1 or 2.5 mg l^{-1}) of 2,4-D or NAA in combination with 0.2 mg l^{-1} TDZ (Table 4.3). Ovule growth was generally low in media containing BAP, which evidently resulted in a high percentage of ovule death. All ovules died in media containing kinetin. Subsequent transfers of healthy ovules to fresh media did not alter their growth patterns, and neither proembryonic callus nor somatic embryos were induced to form on ovules in any of the medium treatments.

4.4.5 Multiple shoot-bud development

Ten percent of zygotic embryos cultured under light (12-hour photoperiod) on MS medium lacking growth regulators germinated into seedlings (Figure 4.9). Multiple shoot-bud development was observed on these germinated seedlings, where approximately 8 shoot-buds were produced per seedling. These multiple-bud shoot developments were, however, not observed on any explants grown in the dark. Shoot-derived plantlets were obtained after removing each shoot-bud from the seedling and rooting them by placing them flat on MS medium in a Petri dish placed upright. Rooting was observed after 4 weeks on MS medium without growth regulators (Figure 4.10A, Figure 4.10B), while callusing of the explants occurred in media containing 1 mg l^{-1} NAA (Figure 4.10C).

4.5 Discussion

Results of this study showed the high competency of *P. cynaroides* zygotic explants to consistently form somatic embryos directly in medium lacking growth regulators, without an intervening callus phase. In addition, these results clearly showed that the addition of exogenous growth regulators, such as NAA, 2,4-D, TDZ, BAP and kinetin inhibited the induction of somatic embryos, while other additives such as casein hydrolysate and coconut water suppressed embryo induction. Inhibition or suppression of embryo induction by growth regulators is rare, since most research papers report the use of some sort of growth regulators (mostly auxins) for the induction of somatic embryos. These results strongly suggest that the source material (zygotic embryos and cotyledons) may be auxin-habituated and are predetermined to somatic embryogenesis (George, 1993).

Results from the direct formation of somatic embryos on zygotic embryos and cotyledons of *P. cynaroides* are in accordance with Sharp, Sondahl, Caldas and Maraffa (1980), where no auxin was required for somatic embryogenesis of the pre-embryogenic determined cells (PEDC). The PEDC were probably determined during a prior mitotic event *in situ*, before their transfer to a cell culture environment. Furthermore, in such cases, the process of embryogenesis is autonomous, and

therefore, once embryo initiation has started, these cells are then able to fulfill their commitment to the embryogenic pattern of development, even in the absence of exogenous growth regulators. These embryos can continue to develop and germinate on the same medium without transfer to fresh medium (Sharp *et al*., 1980).

As mentioned earlier, research papers showing direct somatic embryo formation on growth medium lacking growth regulators are limited. Nevertheless, Gingas and Lineberger (1989) reported that in *Quercus rubra*, the highest percentage of somatic embryos was obtained from immature zygotic embryo tissues cultured on media lacking growth regulators. Similarly, Plata and Viéitez (1990) obtained somatic embryos on MS medium with no growth regulators from cotyledon sections and embryonic axis of *Camellia reticulata*, which completed their development on the same medium.

Although significantly fewer somatic embryos were formed in the dark on explants cultured on MS medium lacking growth regulators (Table 4.1), the ability of the explants to form embryos in both light and dark is noteworthy. This is similar to reports in *Q. rubra*, where embryos formed in both light and dark conditions, however, the highest number of somatic embryos was formed in light (Gingas and Lineberger, 1989). In contrast, high irradiance inhibited embryogenesis in *Glycine max* (Lazzeri, Hildebrand and Collins, 1987). Thus, it is possible that a promotion in the induction of somatic embryos could be affected by a different photoperiod or light intensity. Further research is needed to determine the relationship between different levels of light and the induction of somatic embryos in *P. cynaroides*.

The accumulation of starch grains in parenchyma cells occurred in the vicinity of the developing cotyledons (Figure 4.7). Starch, which is synthesized in the plastids (Esau, 1960), is produced from sucrose supplied in the culture medium (Thorpe, Joy and Leung, 1986). The accumulation of starch grains is known to be a prerequisite for energy-demanding morphogenesis processes (Thorpe and Meier, 1974). Furthermore, starch also acts as a direct cellular reserve of the energy required for morphogenesis, since it disappears rapidly as meristemoids are formed. Therefore the location of the starch grains found in the somatic embryo is an indication that cell differentiation is

taking place, which is seen in the development of cotyledons from the heart stage to the cotyledonary stages (Figure 4.7B; Figure 4.7C).

Gibberellic acid is often used to stimulate the germination of somatic embryos in the post-initiation medium (George, 1993). In this study, the lowest concentration (0.1 mg l^{-1}) of GA_3 promoted the germination of somatic embryos, and as the concentration increased, the germination percentage decreased. The GA_3 concentration that promoted germination in this experiment is similar to that used in other studies. For instance, mostly in the range of 0.3 - 1 mg l^{-1} GA_3, germination of somatic embryos were successful in *Vitis* sp. (Mullins and Srinivasan, 1976), *Citrus* sp. (Kochba, Button, Spiegel-Roy, Bornman and Kochba, 1974) and *Panicum maximum* (Lu and Vasil, 1982). However, there are also numerous papers reporting the germination of somatic embryos in media with no growth regulators, such as in *Olea europaea* (Rugini, 1988), *Dendranthema grandiflora*. (May and Trigiano, 1991), and *Phragmites australis* (Lauzer, Dallaire and Vincent, 2000).

Incidences of malformed embryos were observed in all the medium treatments at the germination stage. The occurrence of malformed embryos seemed to be dependent on the concentration of growth regulators, particularly GA_3, since the lowest concentration of GA_3 (0.1 mg l^{-1}) produced the highest percentage of normal-growing embryos, while as the GA_3 concentration increased, the percentage of embryo malformation in *P. cynaroides* also increased. The malformed embryos were nevertheless able to develop into relatively normal plantlets since most of them possessed deformed cotyledons, while their radicles were intact, which allowed nutrient uptake and further development. A possible remedy to reduce the occurrence of malformed somatic embryos is to incorporate abscisic acid (ABA) into the medium. Manipulation of ABA concentrations has been shown to increase the frequency of embryos to reach maturity (Ammirato, 1988). Its presence in the growth media has been reported to be essential for the normal growth of somatic embryos, and credited with the elimination of abnormal forms of embryos (Ammirato, 1973; Ammirato, 1974). In the current study, we did not find it necessary to approach this method, since the correct concentration of GA_3 was identified to produce healthy, normal plantlets.

In this study, the unsuccessful induction of somatic embryos from unfertilized ovules showed the difference in the requirement of growth media and conditions between zygotes and ovules of *P. cynaroides*. Due to the requirement of complex nutrient media and the sensitivity of ovules to physical conditions, the induction of somatic embryos from unfertilized ovules has often failed (Campion and Alloni, 1990). Among woody plants, somatic embryogenesis has been one of the most thoroughly studied aspects in *Citrus* species (Litz, Moore and Srinivasan, 1985). However, attempts to induce somatic embryogenesis in unfertilized ovules in monoembryonic genotypes have failed (Button and Kochba, 1977; Litz *et al*., 1985). Furthermore, the use of haploid explants to obtain somatic embryos in other plant species has also been relatively unsuccessful. Even in cases where successful induction of somatic embryos from haploid ovules has been reported, the success rates were very low. For instance, for *Allium cepa* (Campion and Alloni, 1990), *Beta vulgaris* (Hosemans and Bossoutrot, 1983) and *Gerbera jamesonii* (Meynet and Sibi, 1984), the yield of somatic embryo induction was only 0.28%, 2.1% and 7%, respectively. These low success rates show that the induction of somatic embryos is very difficult when haploid explants are used. Nevertheless, from the high regenerative capacity shown by the zygotic explants of *P. cynaroides* in this study, it is likely that the regeneration of haploid plantlets from unfertilized ovules can be achieved. Further studies are required to determine their nutrient and environmental requirements such as light and temperature conditions, which are probably the most important factors in culturing these delicate explants.

In the present study, the majority of zygotic embryos formed somatic embryos, while a few germinated into seedlings. However, from those zygotic embryos that did germinate, multiple shoot-bud developments were observed at the cotyledonary node region. This indicates the existence of totipotent cells at this particular region. Although these multiple shoot-buds developed in MS medium without growth regulators, it is often reported that cytokinins such as BAP added singly into the growth medium is required to induce multiple shoot development. Multiple shoot development from embryos has been reported in soybeans (Cheng, Saka and Voqui-Dinh, 1980) and almonds (Hisajima, 1982). Further research is needed to establish the effects of cytokinins on the development of multiple shoot-buds in *P. cynaroides*.

4.6 Conclusion

This study has shown that zygotic explants possess a high competency to form somatic embryos, which can be germinated in medium containing low concentrations of GA_3 in the dark. The establishment of a protocol for direct somatic embryogenesis of *P. cynaroides* improves the prospects of producing *Protea* spp. quickly and efficiently. This is particularly important in a floriculture industry such as proteas where lengthy propagation time and inconsistent rooting is common, partly because seed and vegetative propagation, which have their limitations, are still the most commonly used methods for propagation. The relatively short period of time required to obtain germinated somatic embryos indicate that direct embryogenesis has great potential to be used in the mass production of *P. cynaroides*, as well as in plant breeding research. Further research regarding the establishment of the germinated somatic embryos in *ex vitro* conditions is needed. In addition, the development of multiple shoot-buds on the zygotic explant may be used as an alternative propagation method, but further research is needed.

4.7 References

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Table 4.1. Effects of light conditions on the induction of somatic embryos from zygotic embryos and cotyledons excised from *in vitro*-germinated seedlings, cultured on full-strength growth regulator-free MS medium. Data were taken after 8 weeks in culture.

Explant	Photoperiod	% of explants forming somatic embryos[x]	Mean number of embryos per explants[y]
Zygote	12-h	70.0 a	8.67 ±1.52 a
	24-h Dark	37.5 b	5.53 ±1.49 b
Cotyledon	12-h	25.0 b	4.10 ±1.20 b
	24-h Dark	22.5 b	4.44 ±1.13 b

[x] Data were subjected to Chi-Square analysis. Percentages of 40 explants within column followed by different letters are significantly different at $P \leq 0.001$.
[y] Means within the same column followed by a different letter are significantly different at $P \leq 0.05$ using Tukey's Studentized range test.

Table 4.2. Effects of growth regulators on the germination of somatic embryos cultured in the dark. Half-strength MS medium was used as the basal medium in all treatments. Results were taken after 4 weeks in culture.

GA_3 (mg l^{-1})	Casein hydrolysate (mg l^{-1})	Coconut water (ml l^{-1})	Total germination (%)[x]	Germination of normal embryos (%)
-	-	-	30.0 c	55.6 b
0.1	-	-	83.3 a	72.0 a
0.5	-	-	60.0 b	50.0 b
1	-	-	20.0 c	0 c
2.5	-	-	20.0 c	0 c
5	-	-	20.0 c	0 c
10	-	-	10.0 c	0 c
20	-	-	10.0 c	0 c
-	100	-	20.0 c	0 c
-	200	-	23.3 c	0 c
-	-	50	30.0 c	0 c
-	-	100	40.0 bc	0 c

[x] Percentages of 30 explants within the same column followed by different letters are significantly different at $P \leq 0.001$ according to Chi-square.

Table 4.3. Effects of growth regulators and light conditions on the growth of immature unfertilized ovules after 8 weeks.

Growth medium				Ovule growth (%)	
Half-strength MS + Growth regulators (mg l^{-1})				12-Hour photoperiod	Total darkness
2,4-D	NAA	BAP	TDZ		
-	-	-	-	40.0 c	100 a
1	-	-	-	0.0 d	0.0 d
2.5	-	-	-	0.0 d	63.3 b
5	-	-	-	0.0 d	76.6 b
1	-	0.2	-	0.0 d	0.0 d
2.5	-	0.2	-	0.0 d	53.3 bc
5	-	0.2	-	0.0 d	0.0 d
1	-	-	0.2	0.0 d	73.3 b
2.5	-	-	0.2	0.0 d	60.0 b
5	-	-	0.2	0.0 d	0.0 d
-	1	-	-	0.0 d	30.0 c
-	2.5	-	-	0.0 d	63.3 b
-	5	-	-	0.0 d	53.3 bc
-	1	0.2	-	0.0 d	16.6 c
-	2.5	0.2	-	0.0 d	30.0 c
-	5	0.2	-	0.0 d	0.0 b
-	1	-	0.2	0.0 d	66.6 b
-	2.5	-	0.2	0.0 d	76.6 b
-	5	-	0.2	0.0 d	0.0 d

Percentages followed by different letters are significantly different at $P \leq 0.05$ according to Chi-square.

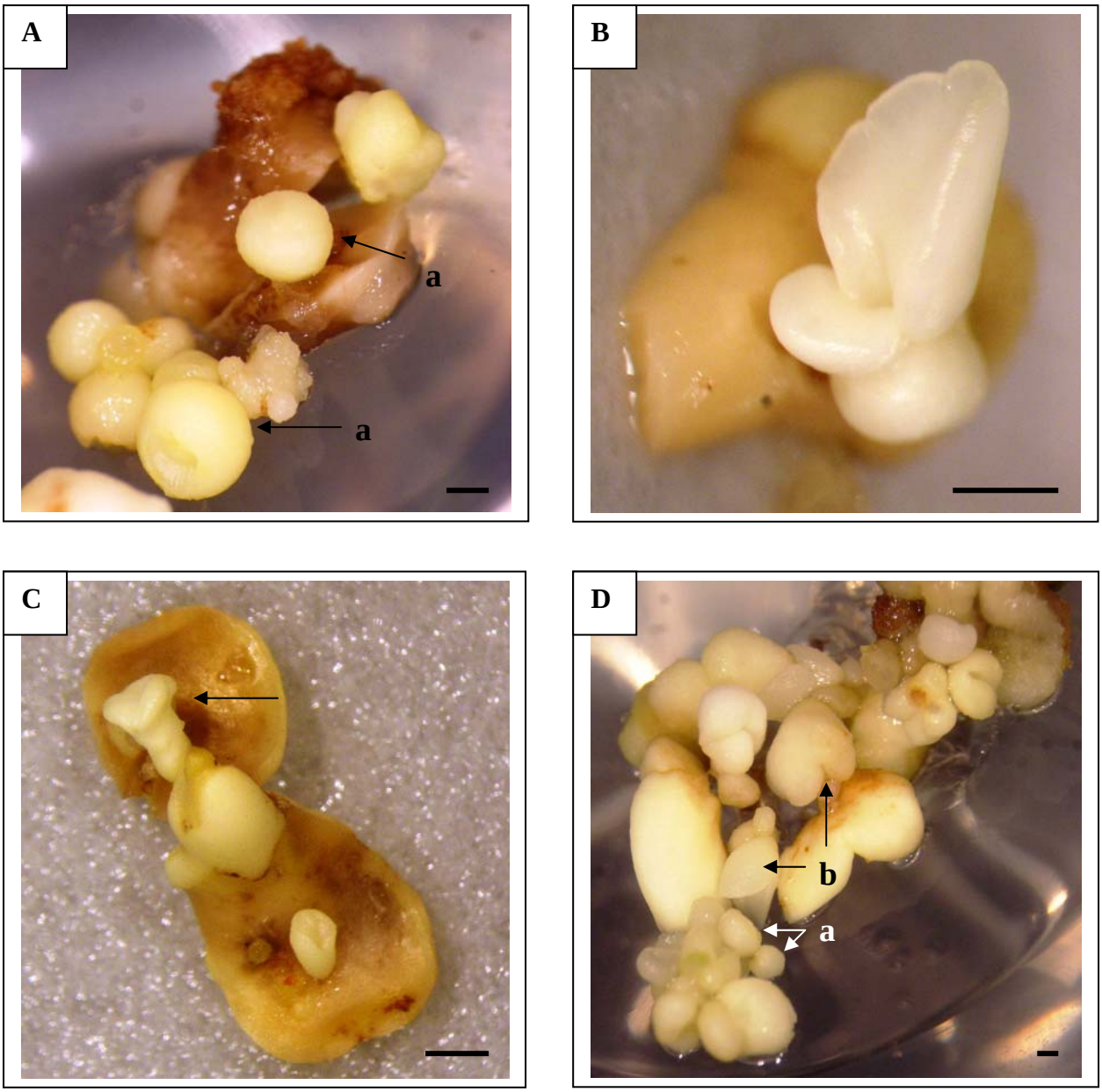

Figure 4.1. Direct formation of somatic embryos: (**A**) globular-stage embryos formed on the edges of a zygotic embryo; (**B**) Advanced torpedo-stage embryo formed on the abaxial side of a zygotic embryo; **(C)** Torpedo-stage embryo formed on the adaxial side of a zygotic embryo (arrow); **(D)** Cluster of somatic embryos from zygotic embryo in various stages of development. **a** = embryos in globular stage; **b** = embryos in heart stage. Bar ≈ 1 mm.

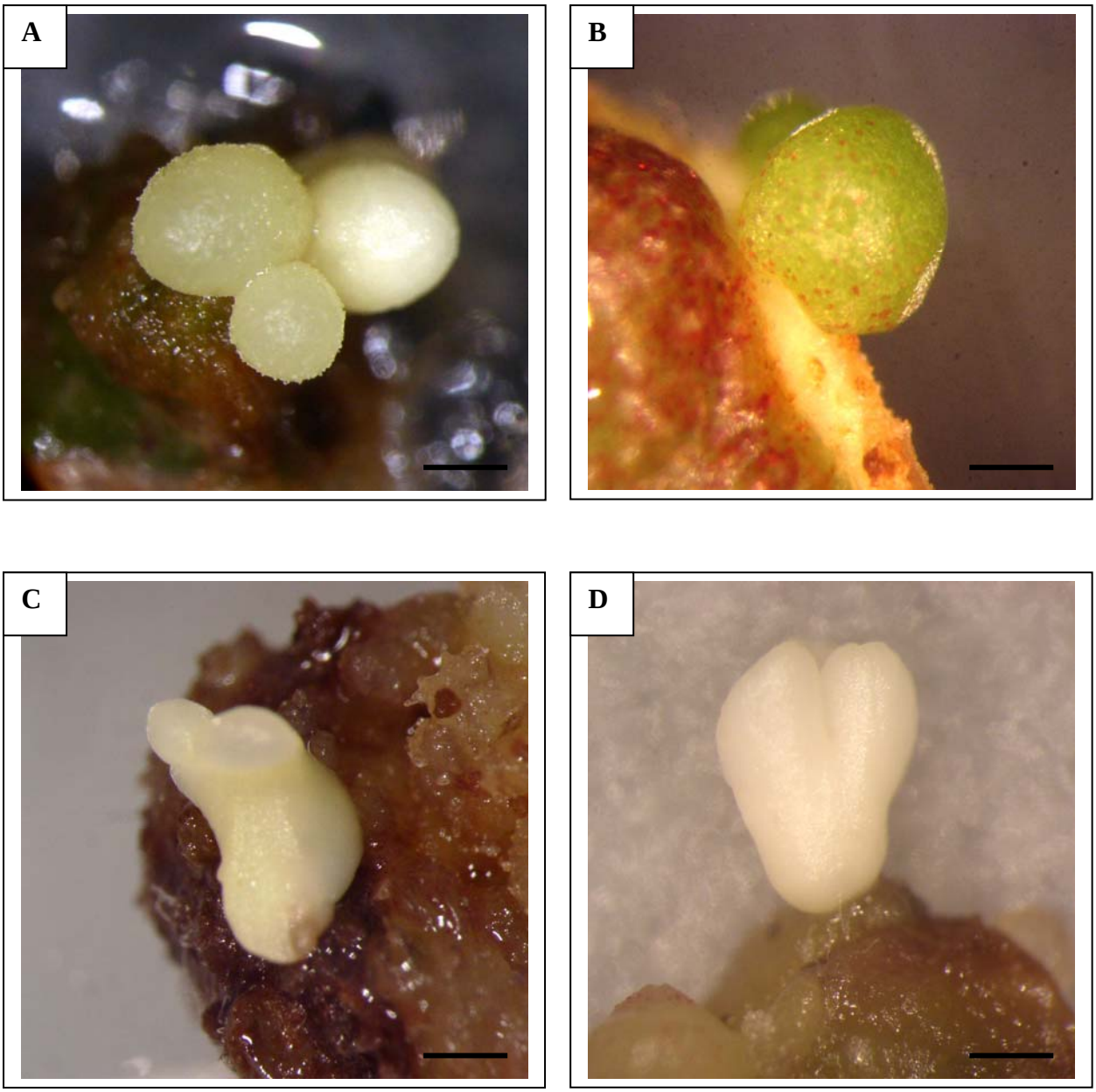

Figure 4.2. Direct somatic embryo formation on excised cotyledons cultured on full-strength MS medium with no growth regulators in **(A)** the dark, and **(B)** under a 12-hour photoperiod. The development of somatic embryo into the **(C)** heart stage, and **(D)** torpedo stage, directly on excised cotyledons in the dark. Bar ≈ 1 mm.

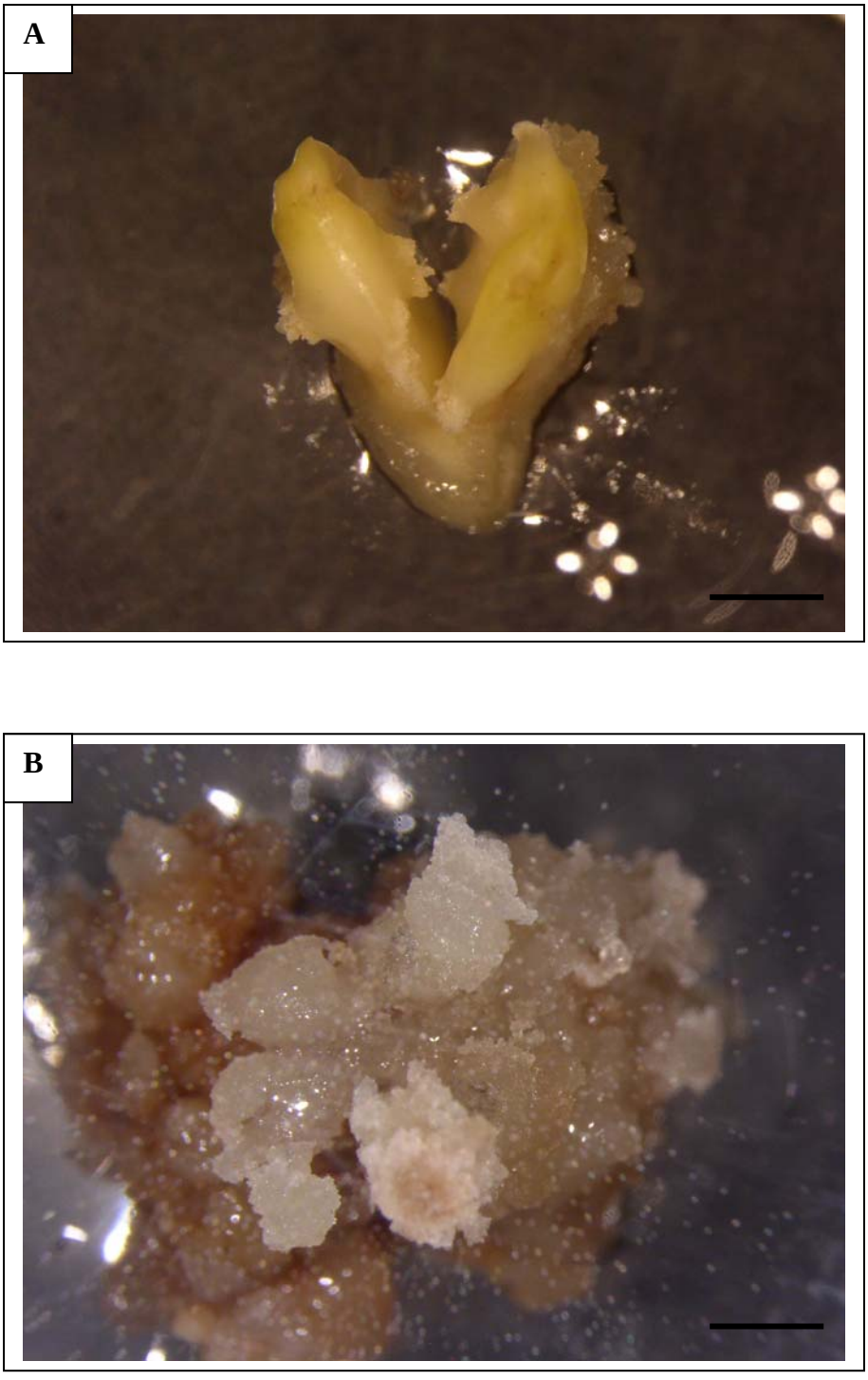

Figure 4.3. Growth of non-embryogenic, watery, fluffy callus on **(A)** zygote explant cultured on medium containing 2.5 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ, and on **(B)** excised cotyledon cultured on medium containing 1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP. Bar ≈ 5 mm.

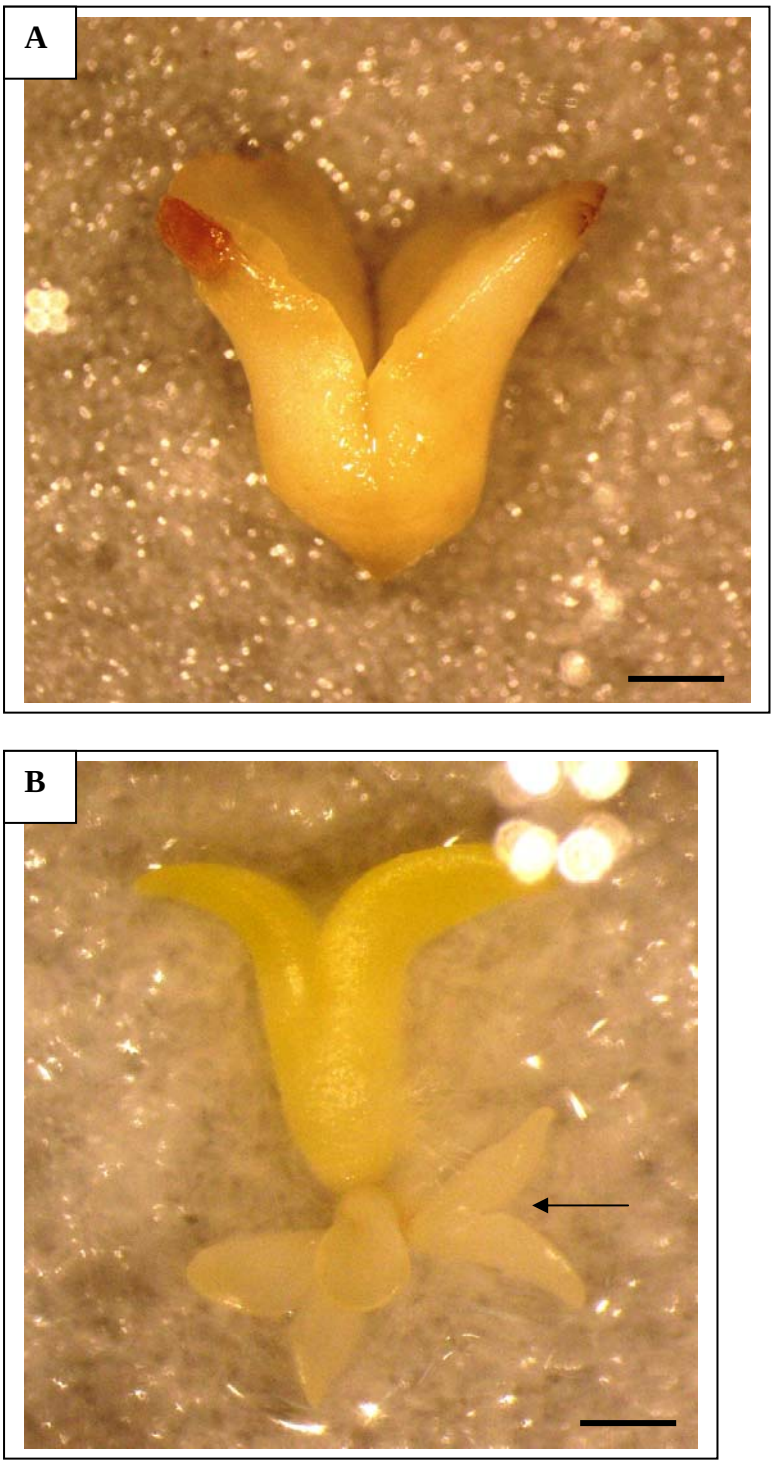

Figure 4.4. Development of isolated somatic embryo cultured on germination medium containing MS medium supplemented with 0.1 mg l^{-1} GA_3. **(A)** Embryo in advanced heart stage; **(B)** Embryo in cotyledonary stage, with accessory embryos attached (arrow). Bar ≈ 1 mm.

Figure 4.5. Germination of normal embryos on MS medium containing GA_3. Emergence of first leaves from the germinated embryo (bottom right). **a** = two separate cotyledons; **b** = radicle. Bar ≈ 5 mm.

Figure 4.6. Germination of malformed embryos with **(A)** single cotyledon; **(B)** multiple cotyledons; **(C)** two fused embryos attached. Bar ≈ 1 mm.

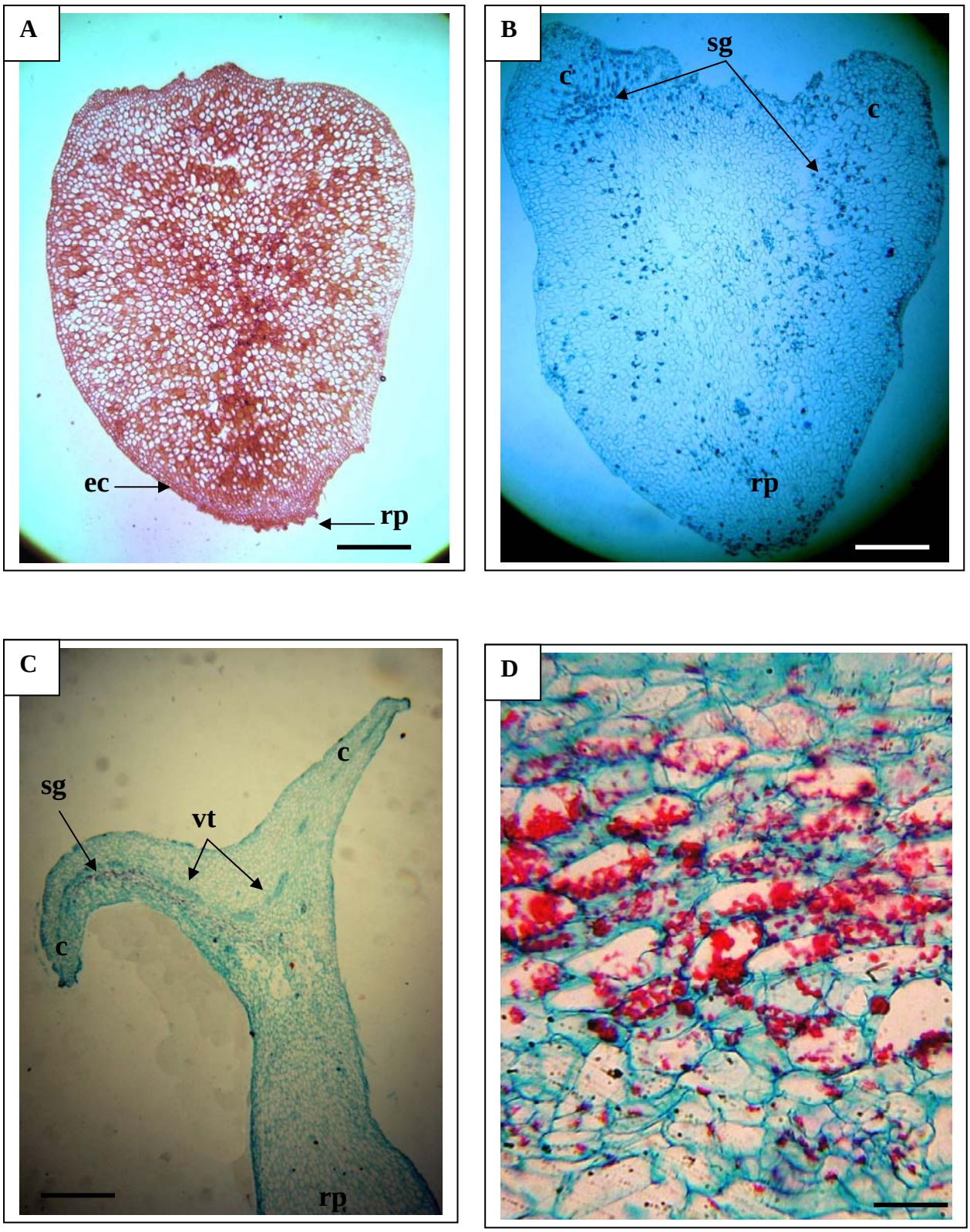

Figure 4.7. Longitudinal section through **(A)** Embryo at globular-stage, with cell differentiation at root pole (Bar ≈ 500 µm); **(B)** Embryo at heart stage, with development of cotyledons (Bar ≈ 500 µm); **(C)** Embryo at cotyledonary stage showing vascular connections (Bar ≈ 500 µm); **(D)** Starch grains stained with safranin, enlarged from the cotyledon area of **(C)** (Bar ≈ 50 µm). **c** = cotyledon; **ec** = epidermal cells; **rp** = radicle pole; **sg** = starch grains; **vt** = vascular tissue.

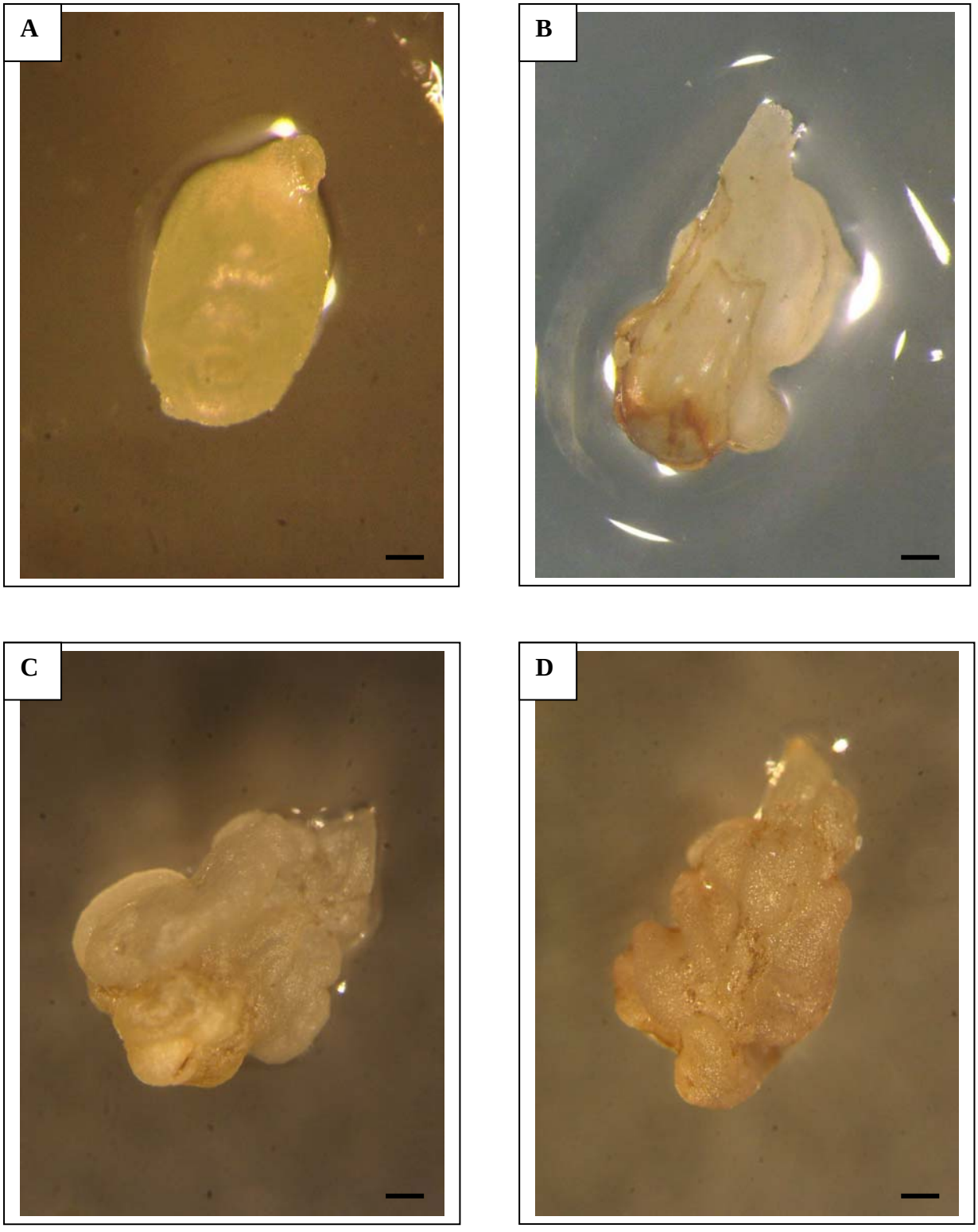

Figure 4.8. Immature unfertilized ovules, cultured on half-strength MS medium with 1 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ after **(A)** 0 days; (**B**) 5 days; **(C)** 4 weeks; **(D)** 8 weeks. Bar ≈ 1 mm.

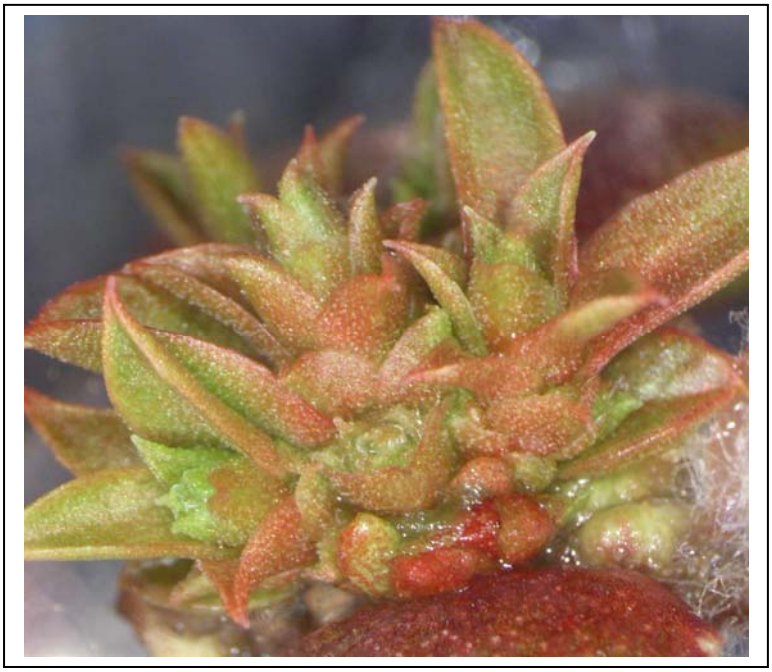

Figure 4.9. Clusters of shoot buds on cotyledons cultured on basal MS medium.

Figure 4.10. (A) Root initiation of excised shoot-bud placed on growth regulator-free MS medium after 4 weeks; **(B)** Root elongation after 6 weeks; **(C)** Callus formation on shoot-bud in 1 mg l^{-1} NAA. Bar ≈ 1 mm.