

**Research Article**

**A Study on the Somatic Embryo Production for *Arnebia Euchroma* Micropropagation**

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**ABSTRACT:**

*Arnebia Euchroma* is medicinal plant of boraginacea family. This genus retains annual and perennial herbaceous species with standing stem, simple and alternate leaves, and bell-shaped and flattened flowers. They are scattered in Asia, Europe and Mediterranean and its smoke from burning it or its poultice were used as disinfectant and for wound healing in traditional medicine. Plant roots contain alkannin, shikonin and their derivatives which are extracted by organic solvents. Micropropagation of this plant through somatic embryo has not been popular. In this research, using seed explants, the direct micropropagation of 10 cultivars of *Arnebia Euchroma* population is studied. Based on the results, *Arnebia Euchroma* seed shows the first signs of callogenesis on the explants were observed after 7 days of inserting the explants in callus induction medium. The seeds inflated and callus formation began afterwards. After 14 days, the callus was easily observable in explants. The calli derived from seed explant were prepared to be returning after 30 days. Derived calli were cream and crunchy.. Before returning, all samples had 100 percent embryogenesis in MS medium. Only calli diameter was studied as the measured factor. On embryogenesis, the MS medium with hormone level of 2.5Mg/L IBA and 2.5Mg/L BA, had the highest callogenesis.

**Keywords:** *Arnebia Euchroma*, somatic embryogenesis, callogenesis

**INTRODUCTION:**

Plants provide the energy and constituents of the body and also the vitamins which regulate the metabolism and the effective materials of the medicines. Using plants for treating diseases has always been a part of human history, boraginacea family is an herbaceous species, and it is hardly found in the form of shrubs and it belongs to the warm and temperate regions. It has coarse hairs and it is one of the best methods of distinguishing this family, from other plant families. It includes 13 genera and 2,200 annual and perennial herbaceous and shrub species. *Arnebia*

*Euchroma*, also known as royle, is of boraginaceae family. This genus has annual and perennial herbaceous species with standing stem, simple and alternate leaves, and bell-shaped and flattened flowers. They are scattered in Asia, Europe and Mediterranean. Pyrrolizidine toxic compounds (ofalkaloids) and N-oxides (which are hepatotoxic and carcinogenic) are considered in toxicology. These compounds are not present in two subfamilies of heliotropioideae and boragunoideae. In traditional medicine, the effective material available in flowers and shoots

of *arnebiaeuchroma* are used for blood purification, softening the chest, strengthening the heart, as a diuretic, diaphoretic and tranquilizer among its other treating properties. Tissue culture is a technique which provides the plant production opportunity *in vitro*. It is based on totipotency which claims that any plant cell includes all genetic data required for converting to a complete plant. Hence, culturing cell, tissue and organ provides the opportunity to form a plant by cultivating one of the aforementioned parts.



**Figure 1**

#### **METHODOLOGY:**

In order to compare plant callogenesis, embryogenesis and regeneration and also the suspension culture, the *arnebiaeuchroma* cultivar which grows in Kerman Province and Aras River banks was used. In each 10 cm Petri plates, 10 sterilized seeds were cultivated on the solid culture medium under sterile conditions. The medium was the basic MS and the complementary organic materials, without hormone and they were kept at  $25\pm 1$  °C and 16 hours of light with intensity of 3,500-4,000 lm. After 4 days, with 2 cm seedlings, the non-polluted Petri plates were opened under sterile condition and their leaves and roots were divided into 2 mm pieces by a scalpel and left in Petri plates containing the basic medium and complementary organic materials with four different hormone levels. Around 10 leaf pieces or

10 root pieces were cultivated in each Petri plate and kept at  $25\pm 1$  °C in the dark. Explants were subcultured in the same medium and under the same conditions after 4 weeks that calli had grown. The measured traits in callogenesis period: Since hundred percent callogenesis took place, the statistical studies in the callogenesis stage was carried out on calli sizes only and after the subculture, the callus volume was measured, using Hooker and Nabors standard (Figure 1).

#### **Embryogenesis Medium:**

When enough amount calli are generated from *arnebiaeuchroma*, they were taken to the embryogenesis medium containing basic media of B5 and MS completed with the following materials:

Medium (1): Without growth regulator materials

Medium (2): 1 MgL 2-4-D + 0.5 MgL BA

Medium (3): 1 MgL 2-4-D + 1 MgL BA

Medium (4): 1 MgL 2-4-D + 2.5 MgL BA

Medium (5): 1 MgL 2-4-D + 5 MgL BA

Medium (6): 2.5 MgL 2-4-D + 0.5 MgL Kin

Medium (7): 2.5 MgL 2-4-D + 1 MgL BA

Medium (8): 2.5 MgL 2-4-D + 2.5 MgL BA

Medium (9): 2.5 MgL 2-4-D + 5 MgL BA

Medium (10): 1 MgL IBA + 0.5 MgL BA

Medium (11): 1 MgL IBA + 1 BA

Medium (12): 1 MgL IBA + 2.5 MgL BA

Medium (13): 1 MgL IBA + 5 MgL BA

Medium (14): 2.5 MgL IBA + 0.5 MgL BA

Medium (15): 2.5 MgL IBA + 1 MgL BA

Medium (16): 2.5 MgL IBA + 2.5 MgL BA

Medium (17): 2.5 MgL IBA + 5 MgL BA

For two weeks, calli were kept in media at  $25\pm 2$  °C for 16 hours of light and 8 hours of darkness and studied based on the embryogenesis rate.

#### **Medium of Converting to Plant:**

In this stage, heart-shaped embryos were kept in the germination medium containing basic SH medium along with hormone compounds of 6 MgL 2-4-D and 0.2 MgL Kin.

#### **Suspension Culture Medium:**

500 mg of embryogenic calli was put in a 100 ml Erlenmeyer flasks containing 20 ml of B5 liquid

medium along with 1 Mg/L of 2-4-D and 0.2 Mg/L of Kin. Erlenmeyer flasks were put on horizontal shakers at 50 rpm in 6/8 hours of light in 5 replications at 27 °C. Subcultures were carried out once each 5 days in the first week and each 10 days during the later subcultures.

After observing the embryo formation in six-week cellular suspension, 1 ml of each medium was deprived by 1 ml sampler and left on the filter and the number of the embryos on the filter in each ml unit was measured.

**Statistical Calculations:**

The required measurements were carried out in the laboratory and the data was analyzed by SPSS and MSTATC software in factorial experiments in completely randomized designs. The correlation coefficient between the data derived from embryogenesis and generation was measured and the interactive effects between various traits were studied and the diagrams were drawn using Excel. Logarithmic transformations were used in all analyses when the inter-treatment variances were not homogenous.

**Table 1** presents the ANOVA of somatic embryogenesis and the number of regenerations which took place in various hormone treatments. It could be observed that there is a significant difference between replications and treatments at one percent level.

	df	Ms	F
Replication	2	35.35	16.17**
Treatment	16	3046.53	1393.48**
E	32	2.18	

In this research, callus was prepared from both root and leaf of *Arnebia Euchroma*. Results are presented in Table 2.

**Table 2.** Callus Induction in *Arnebia Euchroma* from Leaf and Root Explants in various Hormone Compounds

Treatment	2-4-D	IBA	BA	Leaf Callus Induction Percentage	Root Callus Induction Percentage
1	0	0	0	0	0
2	1	-	0.5	8.24	2.21
3	1	-	1	3.37	6.27
4	1	-	2.5	2.47	9.36
5	1	-	5	3.52	8.59
6	2.5	-	0.5	1.68	1.75
7	2.5	-	1	2.90	7.96
8	2.5	-	2.5	1.84	91
9	2.5	-	5	8.83	1.87

**Discussion and Conclusion:**

**Callus Induction Medium:**

On leaf and root explants, around 4 days after leaving the explants in callus induction medium, the first signs of callogenesis were observed on the explants. Initially the root inflated and subsequently, the epidermis was layered and the cells under the epidermis layer began splitting and callogenesis began. After ten days, the callus in explants was easily observed. (Figures 3, 4 and 5) On seed explants, after 7 days of leaving the explants in callus induction medium, the first signs of callogenesis on the explants were observed. Initially, the seeds inflated and subsequently callogenesis began. After 14 days of the callus in explants was easily observed. After 20 days, callus generated from leaf and root explants were ready for installation and the calli derived from seed explant were read for installation after 30 days. The derived calli were cream and crunchy. (Figures 6 and 7) Before installing, since all samples had 100 percent callogenesis in SH medium, only calli diameter was studied as the measured factor.

10	-	1	0.5	9.48	4.33
11	-	1	-	9.64	2.54
12	-	1	2.5	69	2.74
13	-	1	5	5.73	1.83
14	-	2.5	0.5	7.91	5.95
15	-	2.5	1	9.96	7.99
16	-	2.5	2.5	100	100
17	-	2.5	5	7.99	2.89

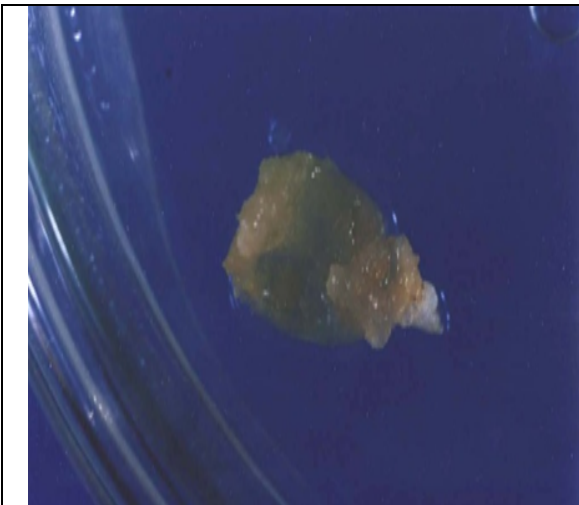
Considering Table 1, callus induction in the first treatment in which no growth regulator was used, no callus induction has taken place. In a similar research conducted by Mangijulaet al. (2005), they reached the same results so that callus induction in hormone-less treatment took place 5 to 20 days after callus induction in all treatments. Callus growth from root explants took around 6 days and callus growth from leaf took round 20 days. The highest rate of callus in root was 100% and in treatment of 2.5 BA+ 2.5 IBA. This situation held true for the leaf; that is, the aforementioned treatment had the highest callus rate in leaf.



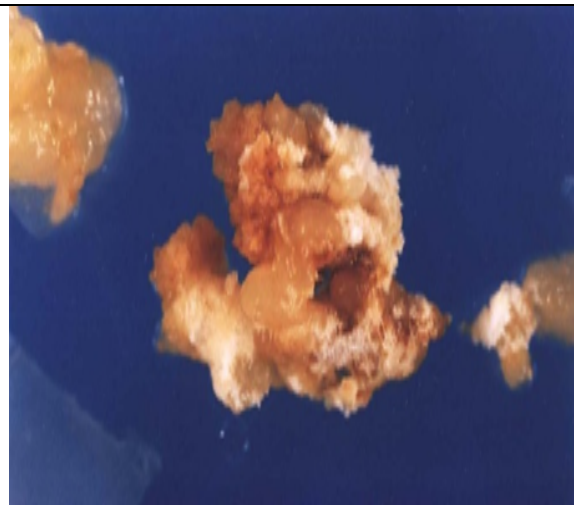
**Figure 2.** Cultivating Sterilized Seeds in Culture with Hormone



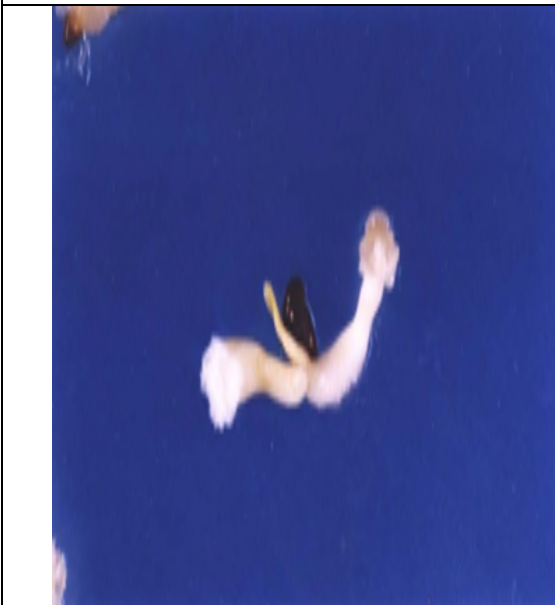
**Figure 3.** Sample of Wet Callus and Juicy Callus



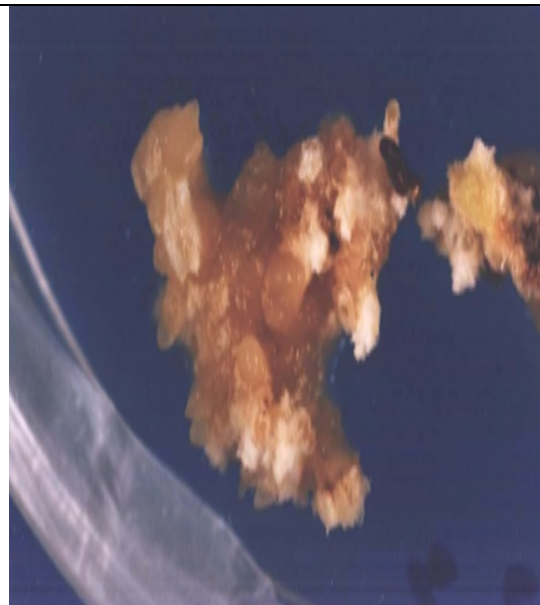
**Figure 4.** Sample of Non-Embryogenic Callus



**Figure 5,** Sample of Embryogenic Callus



**Figure 6.** Initiation of Callus Formation in Seed Explant



**Figure 7.** Sample of Calli Derived from Seed Cultivation

Calli generated from the root were light brown and dense, while the calli derived from leaf were light yellow and less dense, comparing to the root. Root et al. (1998), Leo and Gia (1999) and Mangijula et al. (2005), reached similar results.

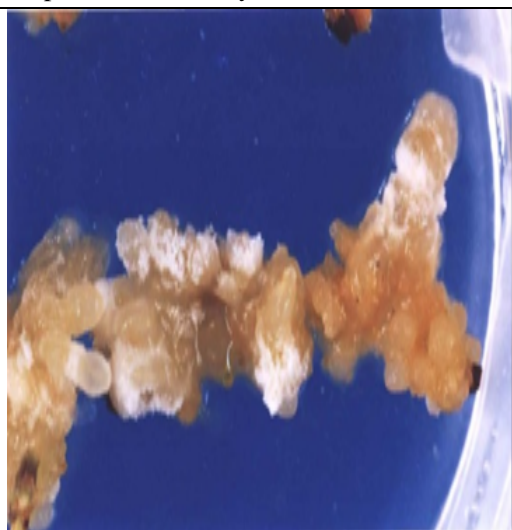
**Regeneration:** Until regeneration signal is not issued, the cultivation medium was changed two times during the four weeks. Regeneration retrieved from leaf calli are green and crunchier, and the regeneration from leaf calli are light brown and denser than the leaf.

Table 3 presents the results from various hormone compounds impact on somatic embryogenesis and shoot organogenesis in *Arnebiaeuchroma* during around 8 weeks after callogenesis.

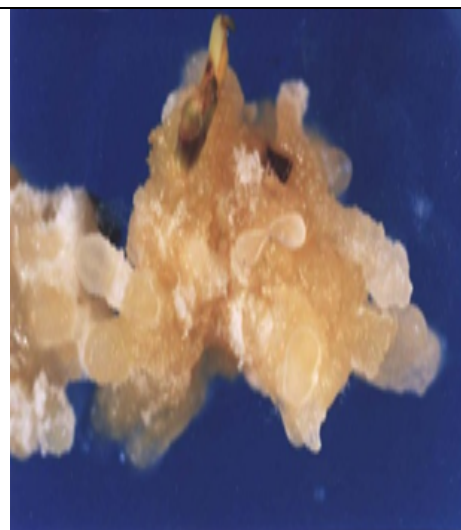
**Table 3.** Results from Various Hormone Compounds Impact on Somatic Embryogenesis and Shoot Organogenesis in *ArnebiaEuchroma* during around 8 Weeks after Callogenesis

Treatment	Somatic Embryogenesis in each Cultivation	Regeneration in each Cultivation	Regenerations
0	-	-	-
9	-	6.1	6.1
10	2.63	9.7	10.5
11	3.6	12.1	15
12	6.4	11.4	17.8
13	11.1	5.6	16.7
14	13.98	4.4	18.38
15	16.2	3.6	19.8
16	8.8	6.2	11.4

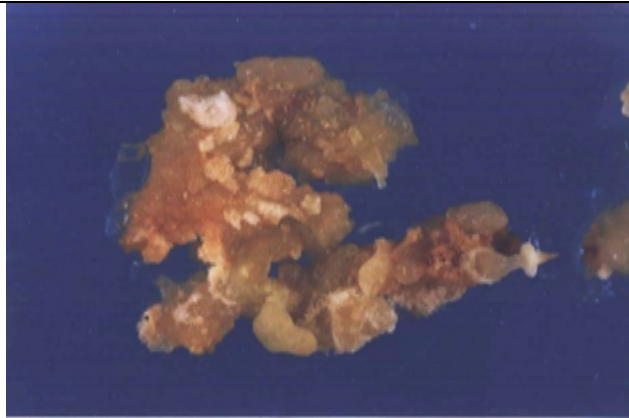
Considering Table 3, it could be observed that based on the number of somatic embryogenesis in each cultivation, the highest rate is related to the treatment 15 with 16.2 and the lowest rate is related to the treatment without any hormone and treatment 9. After treatment 15, treatment 14 had 14 results from various hormone compounds impact on somatic embryogenesis and shoot organogenesis in *arnebiaeuchroma* during around 8 weeks after callogenesis. Based on the number of regenerations in each cultivation, the treatment without any hormone (1) and treatment 12 with 11.4 cases and treatment 11 with 12.1 cases had the highest rank. Yu et al. (1997) reached similar results in their studies on one of *Lithospermumerithrorhyzon* families.



**Figure 8.** Formation of Spherical Embryo



**Figure 9.** Formation of Semi-Spherical Embryo



**Figure 10.** Formation of Spear-Shaped Embryo

**Hormone Level Impact on Embryogenesis:**

Considering table 3, it could be observed that the hormone levels of treatment 15 (2.5 MgL IBA along with 1MgL BA) had the highest impact on embryogenesis and it is categorized in group “a” and other hormone levels, except the hormone level of treatment 14 which contained 2.5 MgL IBA along with 0.5 MgL BA, were in lower groups. These results are in accordance with Mangijulaet al. (2005) results.

**Regeneration Environment:**

After 10 days, the first signs of nodulation (spherical embryos) appeared on callus medium (Figure 8) and after 20 days, the white special embryos turned green and after several days, the spherical embryos transformed into heart-shaped embryos.

**Plant Conversion Medium:**

In this stage, embryos had a spear-shaped form initially and then turned to semi-spherical shape. (Figures 10 and 11) Subsequently, semi-spherical embryos germinated and shoot and root were formed. (Figure 12, 13, 14 and 15)



**Figure 11.** Rooting during Conversion to Seedling



**Figure 12.** Root growth in Plant Conversion Medium



**Figure 13.** Shooting during Conversion to Seedling

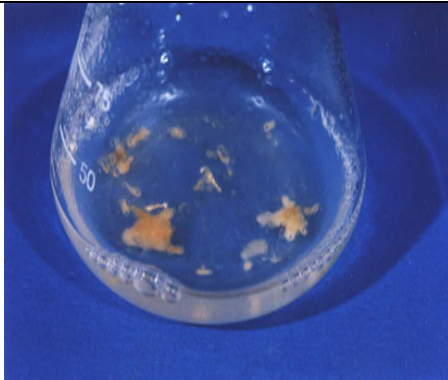


**Figure 14.** Sample of Root and Shoot Formation

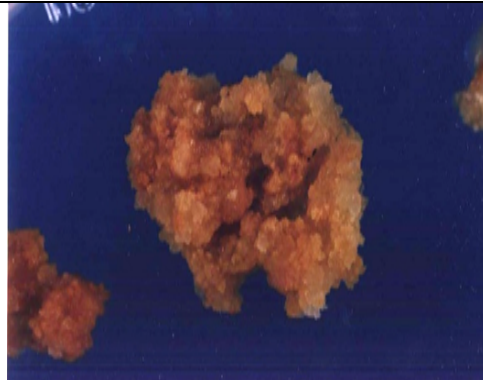
**Suspension Cultivation:**

In this stage, for each Erlenmeyer flask, 500 mg of grown calli was taken to the suspension cultivation medium for embryogenesis.

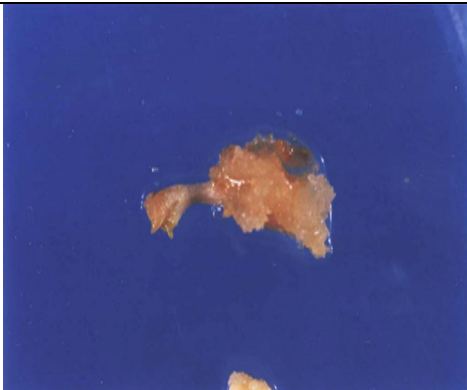
In this stage, 500 mg of embryogenic calli, which were transferred to the 100 ml Erlenmeyer flasks containing 20 ml of liquid B5 medium, containing 1 MgLD-4-2 and 0.2 MgL Kin, was studied. After 10 days, the changes in calli were observed, so that the cells which were separated from each other due to the shaker force were inflated and turned in spherical form. (Figure 6) Accordingly, figures were provided from each of the subculture stages and after 6 weeks after the first cultivation, v in cell suspension were taken for counting under microscope.



**Figure 15.** A Sample of Suspension Cultivation



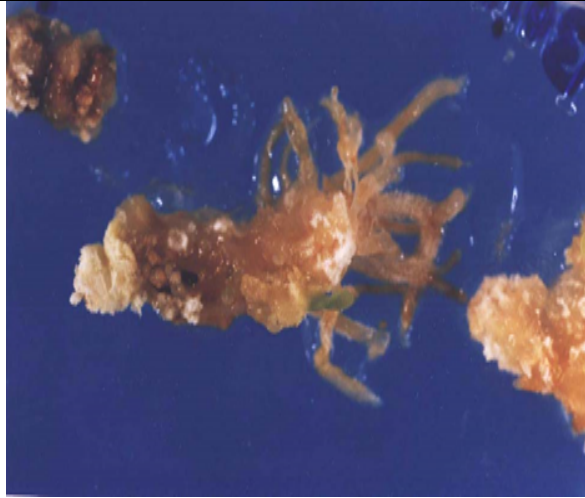
**Figure 16.** A Sample of Callogenic Embryo in Suspension Cultivation



**Figure 17.** germination in Suspension Cultivation Medium



**Figure 18.** Shooting in Suspension Cultivation Medium



**Figure 19.** A Sample of the Embryo development in Suspension Cultivation Medium

#### CONCLUSION

On callogenesis, seed and leaf explant had the highest calli rate. On embryogenesis, the MS medium with hormone level of 2.5 MgL IBA and 2.5 MgL BA, had the highest callogenesis.

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