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# Somatic Embryogenesis and Organogenesis Studies in Tissue Culture of *Onobrichis Altissima* Gross

**Aliheydar Kamalvand [1], Roya Karamian [1], and Parham Jabbarzadeh Kaboli [1,2]***

[1] *Department of Biology, Payame Noor University, 19395-3697 Tehran, Iran*

[2] *Department of Biomedical Science, Faculty of Medicine and Health Sciences, University Putra Malaysia, Selangor, Malaysia*

* Corresponding author's Email: jabbarzadeh@pnu.ac.ir

**ABSTRACT:** With more than 70 species in Iran, *Onobrychis* miller is the biggest genus of the Fabaceae family and is mainly used as fodder due to its nutritional value. For the first time, in this paper, somatic embryogenesis and organogenesis processes are reported using different explants obtained from seedlings (hypocotyle, epicotyle, and cotyledon) of *Onobrychis altissima* species. Different cotyledon, hypocotyle, and epicotyle explants were cultured in the MS (Murashige and Skoog) medium using two concentrations of BAP (6-Benzylaminopurine) and 2, 4- Dichlorophenoxyacetic acid hormones. Frequency of callus induction and new organs were analyzed after two or three subcultures. The results were categorized using Duncan's test. The maximal callus induction on different explants (hypocotyle, epicotyle, and cotyledon) was observed in concentrations of 1 mg and 5 mg per liter of 1-naphthaleneacetic acid (NAA) hormone and concentrations of 0.5 mg/liter and 5 mg/liter of BAP hormone. The largest root induction frequency was observed in MS planting medium having only NAA or low BAP concentrations and maximal germ induction frequency was reported in MS medium with high BAP concentrations compared to NAA treatment.

**Key words:** Somatic Embryogenesis, Organogenesis, Tissue Culture, *Onobrichis*, Fabaceae

ORIGINAL ARTICLE

## INTRODUCTION

With more than 70 species in Iran, *Onobrychis* Miller is the biggest genus of Fabaceae family and is mainly used as fodder due to its nutritional value. *Onobrychis altissima* is mainly found in southwestern Asia, temperate areas, and the Mediterranean region (Aktoklu, 2001).The methods for tissue culture are highly diverse, including somatic embryogenesis (Krikorian and Abraham, 2000). The plant can be distinguished directly by explants and without involvement of callus phase or indirectly after undergoing callus phase (Williams and Maheswaran, 1986). Plant regeneration through somatic embryogenesis has been demonstrated in many herbal species such as *Hyoscyamus acacia*, *Medicago sativa*, and catechu Niger (Rout et al., 2000).

In this paper, somatic embryogenesis and organogenesis processes are reported for the first time using different explants obtained from seedlings (hypocotyle, epicotyle, and cotyledon) in O*nobrychis* species (Sharp et al., 1980; Sharp et al., 1982).

## MATERIALS AND METHODS

After collecting of seeds in the present research, *Onobrichis altissima* gross seeds were initially treated with acid sulfuric. As a result, the seed skin was thinned and germination was accelerated. The seeds were then sterilized by a 20 percent bleach solution. Then, disinfectants were wiped from the seeds following three consecutive washings. The seeds were subsequently cultured in hormone-free MS medium. Cultures were preserved at 25 degrees C. and photoperiod of 16/8 hours of light/darkness. The seeds germinated after two weeks and produced seedlings rapidly. These seedlings were then divided into different 1-cm explants of hypocotyle and epicotyle and cultured in MS medium using different concentrations of two hormones: BAP and 2, 4-D (Fig. 1).

Culturing was performed in three stages. After two to three weeks, calluses were produced from hypocotyle explants in different hormone treatments with different BAP and 2, 4-D concentrations. To induce callus and emergent organs, different explants were transferred into MS medium with different BAP and 2, 4-D concentrations. Callus was produced after three to four weeks. Somatic embryos were moved to hormone-free MS medium for growth, maturation, and regeneration of seedlings (Scarpa, et al., 2000). Statistical analysis was performed using an ANOVA program and the data were categorized by means of Duncan's test.

## RESULTS

An analysis of the effect of different concentrations of 2,4-D and BAP hormones on callus induction in MS medium showed that embryogeneic callus was not formed in 0 and 0.01 mg/liter concentrations of 2,4-D hormone nor in 0, 0.01 and 0.1 mg/liter concentrations of BAP hormone. The evolved calluses were soft and simple. Granular callus was induced in 1 mg/liter and 2 mg/liter

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concentrations of 2,4-D hormone as well as in 1 and 0.1 mg/liter concentrations of BAP hormone (Table 4). Maximal frequency of simple and soft callus inductions (65.6 percent) was observed in 1 mg/liter concentration of BAP and 2,4-D hormones and their minimal induction frequency (2.5 percent) was obtained in 0.01 mg/liter concentration of 2,4-D hormone and 1 mg/liter concentration of BAP hormone (Fig. 1). Following formation of granular callus in different hormone treatments of 2,4-D and BAP, these calluses were transferred to hormone treatments with different NAA and BAP concentrations for inducing somatic embryogenesis and organogenesis. The following results (Tables 1 to 3) can be achieved by comparing different concentrations of NAA and BAP hormones on callus induction in different explants (hypocotyle, epicotyle, and cotyledon). Maximal frequency of callus induction on different explants (hypocotyle, epicotyle, and cotyledon) was achieved in 1 mg/liter and 5 mg/liter concentrations of NAA hormone and 0.5 mg/liter and 5 mg/liter concentrations of BAP hormone. N5-B5 and N1-B0.5 hormone treatments yielded similar effects (Fig. 1 – A).

Through analysis of root induction process in different explants, the largest frequency of root induction was obtained for 1 mg/liter concentration of NAA hormone and 0 mg/liter and 0.5 mg/liter concentrations of BAP hormone. N1-B0 and N1-B0.5 hormone treatments had similar effects and exhibited maximal frequency of root induction (Fig. 1 – B).

Our analysis of germ induction process in different explants showed that N2-B2 and N5-B5 hormone treatments exhibited similar effects. The largest germ induction frequency was achieved for 2 mg/liter and 5 mg/liter concentrations of NAA and BAP hormones (Fig. 1 – A).

Overall, a comparison of results of callus induction in different explants suggests that application of N5-B5 hormone is best for inducing somatic embryogenesis. Maximal frequencies of root induction and germ induction were respectively observed in MS medium having only NAA or low BAP concentrations and MS medium with high BAP concentrations compared to NAA. After transferring spherical somatic embryos to hormone-free MS medium, growth stages of somatic embryos were completed, and, heart-shaped, torpedo shaped, and cotyledon stages and then their germination and seedling formation, were observed (Fig. 2).

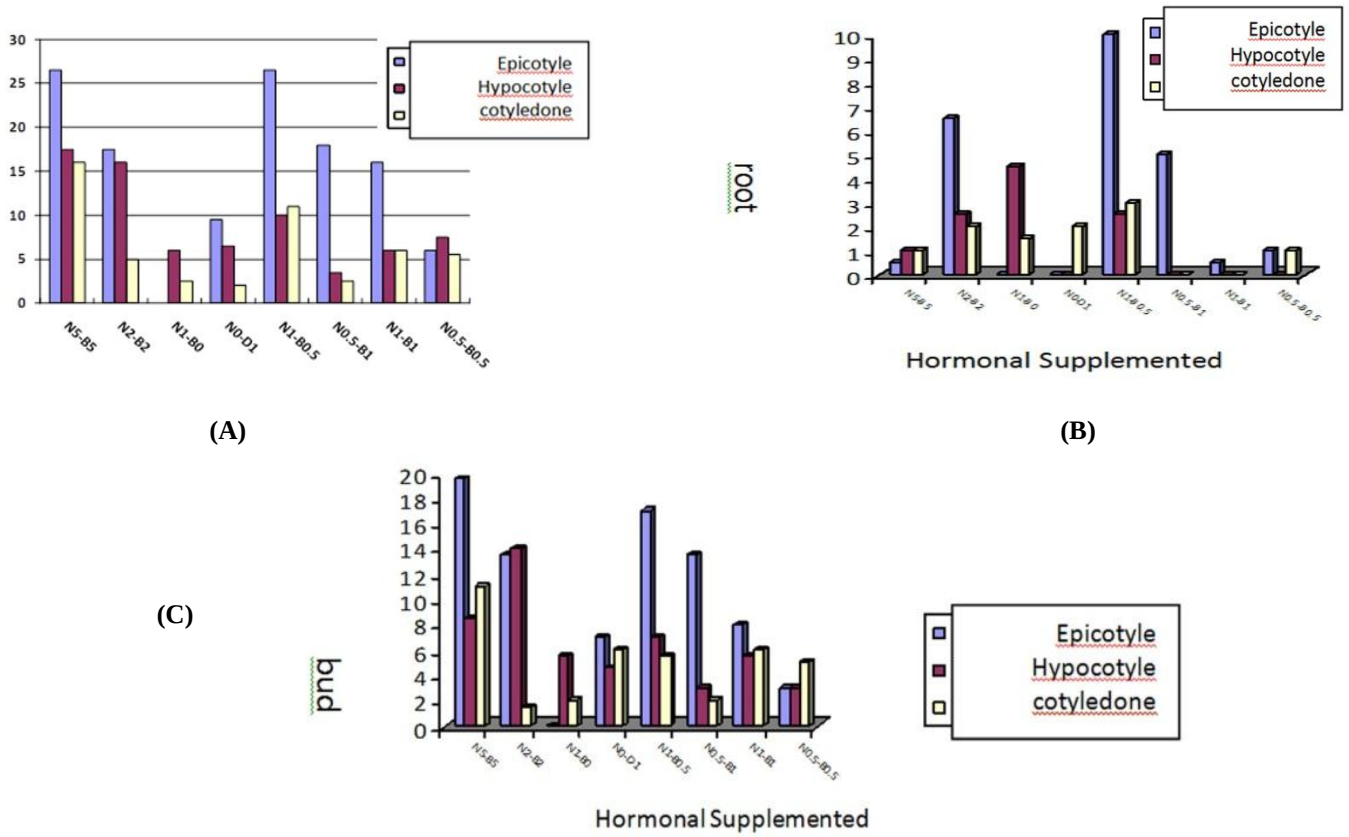


**Figure 1:** Effect of different concentrations of BAP and NAA hormones in formation of embryogeneic callus (1), root formation (2), and germ formation (3) in explants of hypocotyle, epicotyle, and cotyledon explants of *O.altissima*

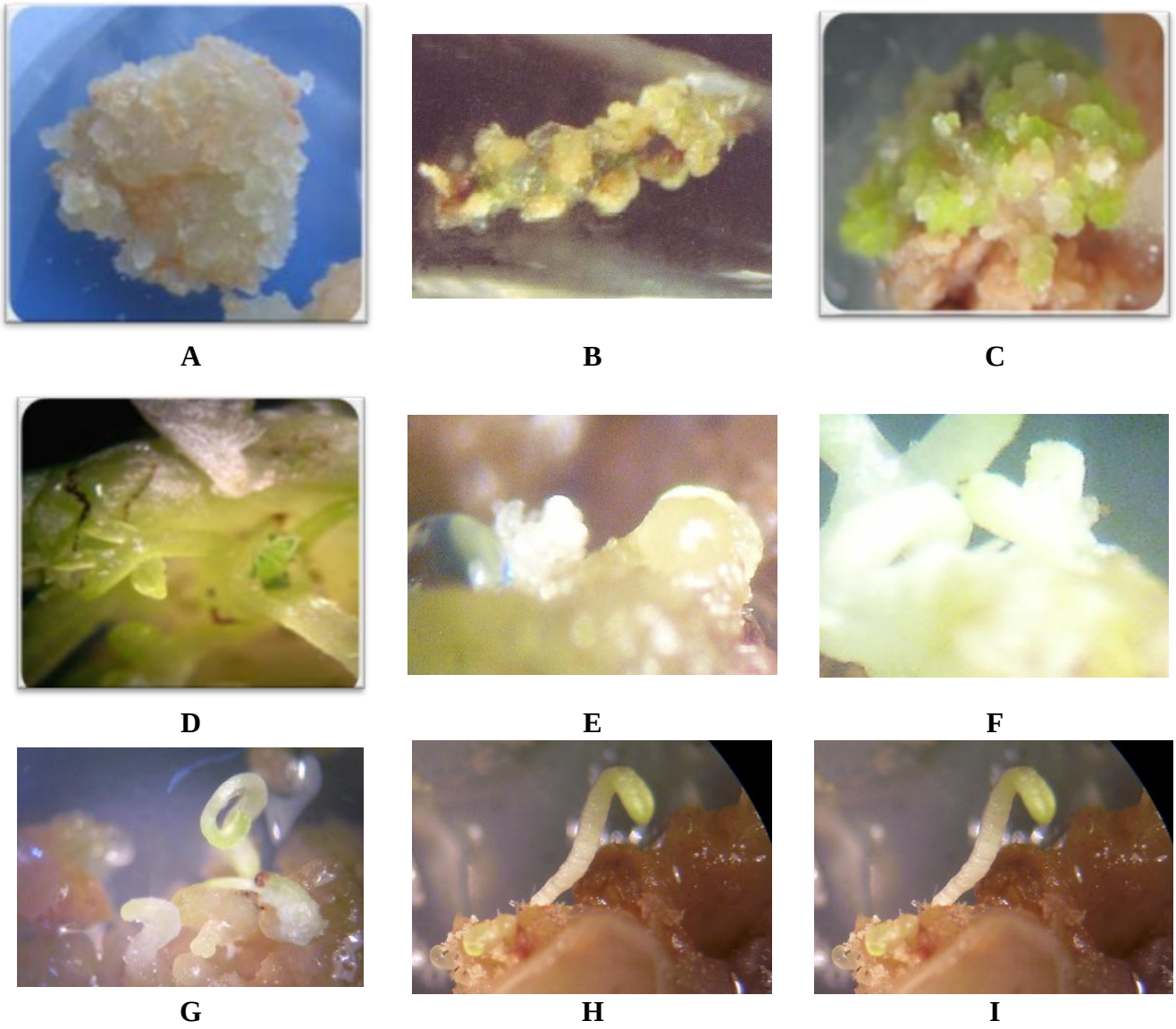


**Figure 2:** Different kind of callus and embryos produced in this research. A: non-embryogenic callus, B: embryogenic callus, C, D: Spherical embryos, E, F: heart-shaped embryo, G,H: germination of embryos, I: seedling regeneration.

**Table 1:** Effect of different NAA and BAP concentrations on organogenesis of cotyledon explants.

| Hormone Treatment | | Embryogeneic callus induction (%) | Root Induction (%) | Germ Induction (%) |
|---|---|---|---|---|
| **NAA(mg/l)** | **BAP** (mg/l) | | | |
| 5 | 5 | 16 ± 1.41 $^{c}$ | 1 ± 0 $^{b}$ | 11 ± 1.41 $^{c}$ |
| 2 | 2 | 5 ± 4.2 $^{a}$ | 2 ± 0 $^{c}$ | 1.5 ± 0.70 $^{a}$ |
| 1 | 0 | 2.5 ± 0.706 $^{a}$ | 1.5 ± 0.70 $^{bc}$ | 2 ± 1.41 $^{a}$ |
| 0 | 1 | 2 ± 0 $^{a}$ | 2 ± 0 $^{c}$ | 6 ± 0 $^{b}$ |
| 1 | 0.5 | 11 ± 1.01 $^{b}$ | 3 ± 0 $^{d}$ | 5.5 ± 2.12 $^{b}$ |
| 0.5 | 1 | 2.5 ± 0.70 $^{a}$ | 0 $^{a}$ | 2 ± 0 $^{a}$ |
| 1 | 1 | 6 ± 1.31 $^{a}$ | 0 $^{a}$ | 6 ± 0 $^{b}$ |
| 0.5 | 0.5 | 5.5 ± 0.70 $^{a}$ | 1 ± 0 $^{b}$ | 5 ± 0 $^{b}$ |

**Table 2:** Effect of different NAA and BAP concentrations on regeneration of epicotyle explants.

| Hormone Treatment | | Embryogeneic callus induction (%) | Root Induction (%) | Germ Induction (%) |
|---|---|---|---|---|
| NAA(mg/l) | BAP (mg/l) | | | |
| 5 | 5 | 26.5 ± 6.36 $^{e}$ | 0.5 ± 0.70 $^{a}$ | 19.5 ± 6.36 $^{d}$ |
| 2 | 2 | 17.5 ± 4.95 $^{d}$ | 6.5 ± 3.41 $^{bc}$ | 13.5 ± 2.12 $^{cd}$ |
| 1 | 0 | 0 $^{a}$ | 0 $^{a}$ | 0 $^{a}$ |
| 0 | 1 | 9.5 ± 0.70 $^{bc}$ | 0 $^{a}$ | 7 ± 0 $^{bc}$ |
| 1 | 0.5 | 26.5 ± 2.12 $^{e}$ | 10 ± 2.83 $^{c}$ | 17 ± 1.26 $^{d}$ |
| 0.5 | 1 | 18 ± 2.83 $^{d}$ | 5 ± 1.12 $^{b}$ | 13.5 ± 0.70 $^{cd}$ |
| 1 | 1 | 16 ± 2.83 $^{cd}$ | 0.5 ± 0.707 $^{a}$ | 8 ± 2.83 $^{bc}$ |
| 0.5 | 0.5 | 6 ± 2.25 $^{ab}$ | 1 ± 0 $^{a}$ | 3 ± 2.83 $^{ab}$ |

**Table 3:** Effect of different NAA and BAP concentrations on somatic embryogenesis and organogenesis of hypocotyle explants

| Hormone Treatment | | Embryogeneic callus induction (%) | Root Induction (%) | Germ Induction (%) |
|---|---|---|---|---|
| NAA(mg/l) | BAP (mg/l) | | | |
| 5 | 5 | 17.5 ± 6.36 $^{b}$ | 1 ± 0 $^{ab}$ | 0.5 ± 0.70 $^{d}$ |
| 2 | 2 | 16 ± 4.2 $^{b}$ | 2.5 ± 0.07 | 14 ± 0 $^{e}$ |
| 1 | 0 | 6 ± 1.41 $^{a}$ | 4.5 ± 0.70 $^{c}$ | 5.5 ± 0.70 $^{bc}$ |
| 0 | 1 | 6.5 ± 2.12 $^{a}$ | 0 $^{a}$ | 4.5 ± 0.70 $^{ab}$ |
| 1 | 0.5 | 10 ± 0 $^{ab}$ | 2.5 ± 2.12 $^{b}$ | 7 ± 1.41 $^{cd}$ |
| 0.5 | 1 | 0.5 ± 0.70 $^{a}$ | 0 $^{a}$ | 3 ± 0 $^{a}$ |
| 1 | 1 | 6 ± 0 $^{a}$ | 0 $^{a}$ | 5.5 ± 0.7 $^{bc}$ |
| 0.5 | 0.5 | 7.5 ± 2.12 $^{a}$ | 0 $^{a}$ | 3 ± 0 $^{a}$ |

**Table 4:** Effect of different 2,4-D and BAP concentrations on granular callus induction of hypocotyle in Onobrychis altissima

| Granular callus induction (%) | Hormone treatment | |
|---|---|---|
| | BAP mg/l | 2,4-D mg/l |
| 0 | 0 | 0 |
| 0 | 0.01 | 0.01 |
| 0 | 0.1 | 0.01 |
| 13.7 ± 1.6 bc | 1 | 0.01 |
| 2.5 ± 0.3 d | 0.01 | 0.1 |
| 27.8 ± 2.1 b | 0.1 | 0.1 |
| 37.14 ± 5.7 b | 1 | 0.1 |
| 50.4 ± 1.3 b | 0.01 | 1 |
| 61.0 ± 3.1 a | 0.1 | 1 |
| 65.6 ± 3.2 a | 1 | 1 |
| 54.6 ± 3.2 ab | 2 | 1 |
| 52.9 ± 1.8 ab | 1 | 2 |

*The values in each column written with different letters represent significant difference at probability value of P=0.05.

## DISCUSSION

The current research results align with those obtained from culturing hypocotyle explants of onobrychis subniten in MS medium with different 2,4-D and BAP concentrations. In terms of germ formation, no significant difference is observed among different explants, and all explants have branching ability. In all treatments, germs were produced indirectly and after callus induction. The largest germ induction in explants was observed in higher NAA concentrations up to 2 mg/liter and 5 mg/liter. For fixed NAA concentration, an increase in BAP concentration is followed by further germ induction.

Results of the study conducted by Snanck (1999) on micro-propagation of *onobrychis viciifolia* implied that the maximal new twig-induction frequency is observed on epicotyle explants in direct cases and without undergoing a callus phase. If the ratio of BAP to NAA concentrations is 20 to 40, reduction of this ratio directly reduces frequency of new twigs and their length.

Results on *O. subnitens* in former studies (Evans, D.A., Sharp, W. R., and Flick, C. E., 1981; Aktoklu, 2001) suggested that the greatest frequency of indirect new twig induction is achieved on hypocotyle explants in BAP to NAA concentration ratios of 5-10. In the current study, maximal frequency of embryogeneic callus induction on hypocotyle explants of O. subnitens was reported in BAP to NAA ratios of 1 or 2. In another genus of legume, "*Astragalus melilotoides*," maximal frequency of embryogeneic callus induction was reported in BAP to NAA ratios of 2 (Huang et al., 2004). A transfer of embryogeneic calluses to hormone-free medium led to different maturation stages of somatic embryos and finally seedling regeneration. A positive effect of hormone elimination from culture medium on the maturation of somatic embryos was reported in another species of onobrychis subnitens and also some other legumes (Hun and Jia, 2004; Huang, et al., 2005).

The results of culturing different explants of *Onobrychis altissima* in MS medium using different concentrations of BAP and 2,4-D hormones showed that these treatments are highly effective in callus induction, but had little impact on organogenesis (Monash, et al., 2004). The calluses produced in these treatments primarily lacked any sort of morphogenetic potential. 2,4-D hormone in concentration of 1 mg/liter demonstrated a maximal effect on induction of granular and embryogeneic calluses. A concentration of 2 mg/liter of this hormone yielded the largest impact. Among the explants, hypocotyle-induced embryogeneic callus and other explants only yielded simple calluses without organogenesis ability (Mesella, et al., 2005). Furthermore, the results of culturing different *O.altissima* explants in MS medium with varied concentrations of NAA and BAP hormones showed that all explants in most treatments produce callus at higher frequencies. If the medium lacks one of the above hormones (NAA and BAP) increasing in number of callus inductions shortened epicotyle length. The negative impact of high BAP concentrations on induction of embryogeneic calluses is further evident particularly in low NAA concentrations. The maximal embryogeneic callus induction is mainly observed in epicotyle explants and in equal proportions of NAA and BAP hormones. Induction of embryogeneic callus was observed in hypocotyle explants, and next, in cotyledon with lower frequency. Maximal root induction frequency in different explants, especially hypocotyle, was achieved in high NAA concentrations up to 1-2 mg/liter. Negative results were observed in higher concentrations. In fixed NAA concentration, an increase in BAP concentration resulted in less root induction.

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