

# Hormonal Regulation of Somatic Embryogenesis and Organogenesis in *Alyssum borzaeanum* Nyár. *in vitro* Culture System

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## Abstract

Among the tools of modern biotechnologies tissue culture is being increasingly applied to complement other *in-situ* or *ex-situ* conservation methods. Within the Institute of Biology from Bucharest, several *in vitro* techniques have been developed, with recent establishment of endangered plant tissue culture collections. Hormonal regulation of the regeneration process was histologically monitored in order to develop for the first time, a protocol for efficient multiplication and establishment of an *in vitro* collection of *Alyssum borzaeanum* germplasm. The most efficient hormonal combination for shoot regeneration was 4.43  $\mu$ M BAP and 0.57  $\mu$ M IAA. The described correlations between morphogenetic pattern and growth regulators type and content enable the control upon the development processes being useful for further investigation and use of *Alyssum* species.

**Keywords:** *Alyssum borzaeanum*; *in vitro* culture; hormonal control; conservation.

## 1. Introduction

The dramatic loss of plant genetic resources from last two decades has made necessary the development of new *ex situ* conservation approaches. Biotechnological methods like *in vitro* tissue cultures offer a valuable *ex situ* conservation alternative to complement *in situ* methods.

*Alyssum borzaeanum* is an endangered endemic plant with a limited distribution along the coast of Black Sea. It was first mentioned in Romania by E.I.Nyárády [1]. It was also cited in Ukraine [2], Turkey, Northern Greece [3], and Bulgaria, where it is classified as *A.tortuosum* Willd. subsp.*borzaeanum* (Nyár.) Stoj. & Stef. [4]. In Romania it is found only in the Constanta district, between Tuzla and the seaside. In the *Red List of Higher Plants from Romania* [5], *A.borzaeanum* is classified in the *endangered* zoologic category, and since 1998 it has been included in *Annex I* of the *Bern Convention*. Apart of the conservation interest, *Alyssum* species are investigated mainly because of their phytoremediation potential by

nickel hyperaccumulation [6-9]. According to Kramer et al. [10] there are more than 400 hyperaccumulating species among which 48 belong to *Alyssum* genus. Furthermore a number of *Alyssum* species are used as a traditional herbal medicine, especially seeds that are known to contain a large amount of bioactive substances recommended by herbalists for coughs, asthma, lung infections, demulcent and kidney stone [11, 12]. Previous methods of *ex-situ* culture of *Alyssum* species were developed to investigate their nickel hyperaccumulation capacity [6, 8]. There is a single report upon *A. borzaeanum* somatic embryogenesis and the use of *in vitro* regeneration potential for conservation purposes [13]. Hormonal regulation of the explant morphogenetic response (embryogenesis or organogenesis) is very complex and varies widely between species of the same genus and sometimes between the varieties of the same species. In this context the studies targeted to specific taxa are of peculiar importance. In the present work hormonal effect upon plant regeneration was histologically monitored in order to select the most efficient *in vitro* tissue culture protocol. The described methods offer a valuable alternative for *ex situ* conservation of this endangered endemic plant and could be also applied to other *Alyssum* species for conservative, phytoremediation, bioactive substances extracts or other purposes.

## 2. Methods

### Plant material, explants preparation and culture media.

Mature seeds collected from Agigea Natural Reservation were surface disinfected in 70% ethanol for 1 minute, followed by 8 minutes in 0.5% (w/v) Sodium dichloroisocyanurate and washed three times in double distilled sterile water. Seeds were sterile inoculated on basal Murashige and Skoog (MS) medium [14], without growth regulators and were incubated at 20°C in dark condition. Four days old seedlings were used as explant source. Explants (fragments of cotyledons and hypocotyls about 3-5 mm long) were placed on MS medium supplemented with growth regulators as shown in

Table 1. Each variant consisted of 10 explants and three repetitions per variant were used. MS medium without growth regulators was used as control.

Established cultures were maintained at 20°C with a 16 h photoperiod.

After 8 weeks of culture well developed shoots were transferred for root regeneration to MS medium without growth regulators. After 4 weeks the plantlets with well-established roots were transplanted to autoclaved soil in pots and watered for 10 days with a half-strength MS solution containing 2% sucrose. Micropropagated plantlets were successfully established in soil with 85% survival rate after hardening.

**Table1.** Growth regulators content of culture media.

Variant	Auxin	Content ( $\mu$ M)	Cytokinin	Content ( $\mu$ M)
M0	-	-	-	-
M1	NAA	0.53	BAP	4.43
M2	NOA	0.49	BAP	4.43
M3	IAA	0.57	BAP	4.43
M4	IBA	0.49	BAP	4.43
M5	2,4D	0.45	BAP	4.43
M6	2,4,5 T	0.39	BAP	4.43
M7	NAA	0.53	Kn	4.64
M8	NOA	0.40	Kn	4.64
M9	IAA	0.57	Kn	4.64
M10	IBA	0.49	Kn	4.64
M11	2,4D	0.45	Kn	4.64
M12	2,4,5 T	0.39	Kn	4.64
M13	2,4D	4.52	Kn	0.92
M14	IAA	14.25	Kn	0.09
M15	NAA	13.42	BAP	0.04
M16	2,4D	11.30	-	-

Note: **BAP** – 6-benzylaminopurine,

**IAA** – Indole-3-acetic acid,

**IBA** – Indole-3-butyric acid,

**Kn** – Kinetin (6-furfurylaminopurine),

**NAA** – 1-naphthylacetic acid,

**NOA** – 1-naphthoxyacetic acid,

**2,4D** – 2,4-dichlorophenoxyacetic acid,

**2,4,5T** - 2,4,5- trichlorophenoxyacetic acid.

### Histological studies

Fresh tissue samples in different developmental stages were fixed for 2 weeks in a formalin buffered solution (15% (v/v) formalin, 2% (w/v) sodium bicarbonate). Dehydration was conducted at room temperature in graded ethanol series (5 to 100%), followed by clearing with xylene and embedding in paraffin according to usual procedures [15]. Serial sections (7-10 $\mu$ m thick) were cut using a rotary microtome and transfer onto glass slides. Paraffin was removed and the sections were stained with 0.12% (w/v) toluidine blue, in a water solution containing 5% (w/v) sodium tetraborate decahydrate and 0.1% (w/v) basic fuchsin [16] and finally mounted in synthetic resin (Entallan). Histological

observations were made using bright-field light microscopy under a Nikon Eclipse E200 microscope and micrographs were recorded using a Nikon Coolpix 5400 digital camera.

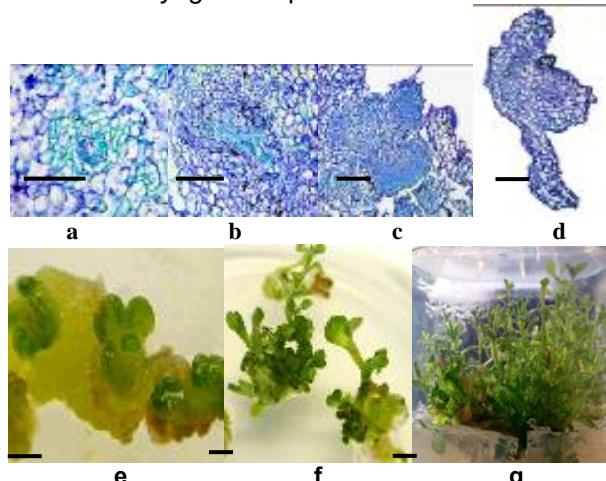
### 3. Results and Discussion

The use of combinations of growth regulators (auxins and cytokinins) induced a range of developmental responses. Within 30 days of culture explants shows 6 distinct types of responses, as follows:

1 – **NM** (*Nonmorphogenetic*) – Explants were swelling progressively without any modification of epidermal cells. Cultures on fresh media did not result in any morphological events. After more than 40 days of cultures the swollen explants become necrotic and degenerate.

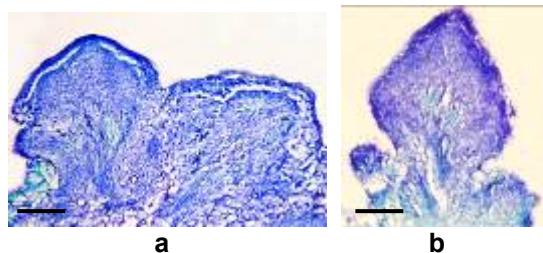
2 – **C** (*Callogenesis*) – Explants were disorganized by intense divisions of mesophyll cells resulting in a white smooth callus without morphogenetic potential. In cultures for longer than 30 days calli turned brown and gradually degenerated and died.

3 – **ISE** – Explants were disorganized by intense divisions of both external and internal tissues resulting in a rough callus with a number of mitotic active structures. After 45 days of culture, the proembryonic structures developed somatic embryos and finally shoots and plantlets (Figure 1). This regeneration pathway corresponds to *indirect somatic embryogenesis* process.



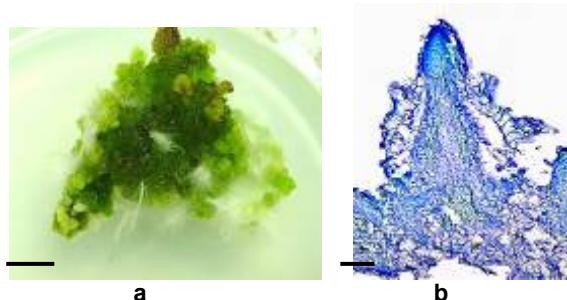
**Figure 1.** ISE- type response (Indirect somatic embryogenesis); **a-d** - stages of somatic embryos maturation, **e** – shoot primordia, **f** – shoot, **g** – plantlets (bar scale = 100 $\mu$ m for **a-d**, 1mm for **e,f**, 2mm for **g**)

4 – **DSE** – Some cells from epidermis and subepidermis were active dividing result in embryogenic precursor structures which dedifferentiated into somatic embryos (Figure 2). Within 40 days of culture the embryos were matured and germinated generating plantlets. This pathway characterize *direct somatic embryogenesis* process



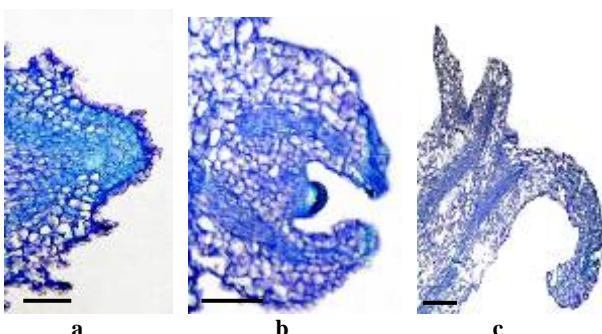
**Figure 2. DSE - type response (Direct somatic embryogenesis); a - embryogenic structures, b – emerging embryo (bar scale = 100μm)**

5 – **R** (*Rhizogenesis*) – Explants are disorganized progressively by intense cell proliferation resulting in a compact callus from which, after 35 days of culture were differentiated radicular primordium (Figure 3). Culture more than 40 days did not show any other morphogenic response.



**Figure 3. R- type response (Rhizogenesis); a – rhizogenic callus, b – radicular primordium (bar scale = 0.5 cm for a, 100μm for b)**

6 – **DO** – Explants are slightly swelling and after 25-30 days of culture, adventitious buds and shoots were developed from meristematic structures (caulinar meristem-like structures) as the result of the mitotic activity of competent epidermal and parenchymatous cells (Figure 4). This morphogenic response corresponds to *direct organogenesis*.



**Figure 4. DO- type response (Direct organogenesis); a-caulinar meristems-like structure, b – adventitious bud, c – adventitious shoot (bar scale = 100μm)**

Based on the present results the main factors conditioning somatic morphogenesis in *A. borzaeanum*

are the composition of the culture media, especially the growth regulators and the morphogenic competence of some of the explant cells. The explant response type to culture media hormonal content was summarized in Table 2.

**Table2.** Explant response to hormonal content of culture media, after 5 weeks of culture.

Media variant	Response type	No. of shoots / Explant <sup>a</sup>	Mean length of shoots / explant (mm)
M1	ISE	9.8±0.8	17.3±0.6
M2	ISE	14.9±0.6	12.2±0.83
M3	ISE	17.1±0.5	22.4±0.3
M4	ISE	11.3±0.3	7.2±0.4
M5	ISE	1.6±0.1	2.8±0.3
M6	ISE	7.2±0.4	8.1±0.4
M7	ISE	3.1±0.3	4.2±0.6
M8	DO	5.7±0.3	7.3±0.4
M9	ISE	9.3±0.4	12.3±0.3
M10	ISE	7.2±0.3	11.8±0.4
M11	ISE	1.6±0.1	1.3±0.1
M12	ISE	2.7±0.3	1.8±0.1
M13	DSE	4.8±0.1	4.1±0.3
M14	R	0.0	0.0
M15	R	0.0	0.0
M16	C	0.0	0.0
M0	NM	0.0	0.0

Note: <sup>a</sup>Values are means ± standard errors of variants with three replicates, each variant consisting of a total of 30 explants; Abbreviations are explained in text.

The combined content in both auxins and cytokinins of culture media influenced the morphogenetic competence of the explant cells, the most frequent response being indirect somatic embryogenesis (ISE). Although concentrations of cytokinins less than 5  $\mu$ M combined with low concentrations of auxins (< 0.5  $\mu$ M) favoured embryogenic callus development and shoot regeneration, not all the combinations were efficient in high rate shoot production. Low concentrations of some auxins like 2,4D and 2,4,5T, caused inhibition of embryo development and favoured callus formation, showed by the lower shoot regeneration on M5, M6, M11 and M12 media. This results confirm the inhibitory role of 2,4 D upon somatic embryo development previously reported in other brassicas [17]. The most efficient combination with best results in terms of number of shoots per explant (17.1) was 4.43  $\mu$ M BAP and 0.57 IAA on M3 media. Despite previous findings to other genus of *Brassicaceae* [18, 19, 20] BAP was more efficient than Kin and IAA than NAA, respectively, in *A. borzaeanum* shoot regeneration.

Direct somatic embryogenesis was achieved on media with 4.52  $\mu$ M 2,4D and 0.92  $\mu$ M Kin suggesting that concentration about 5  $\mu$ M 2,4 promote proembryonic masses formation from competent epidermal and parenchymatous cells and Kin content of about 1  $\mu$ M stimulate somatic embryo development and shoot regeneration.

Direct organogenesis was induced on M8 media only, meaning that auxin NOA combined with Kin were simulative to dedifferentiation process of some competent cells of the explant.

Regeneration under single auxin effect (2,4 D) failed, the only response being callus formation (C type response). This type of the explants response confirms the callusogenic effect of 2,4D reported in a number of plant species including brassicas [17,18].

Cultivation on media with dominant auxin and low cytokinin content (M14 and M15) lead to regeneration of roots, rhizogenesis being the only morphogenetic response. Auxins (IAA and NAA) were promoting callogenesis and cytokinins (Kin and BAP) rhizogenesis.

It was also found that basal media without growth regulators (M0) failed to induce any developmental response of the explants, as well as previous reported on *Eruca sativa* [17] and *Camelina sativa* [19].

#### 4 Conclusions

Histological monitoring of the explant response to *In vitro* culture conditions reveals the morphogenetic pathway and allows an optimal establishment of culture protocol according to a specific purpose. If *A. borzaeanum* vitroculture system is established for germplasm conservation or for phytoremediation potential investigation, is recommended media formulation which induces shoot regeneration through direct (M8) or indirect pathway (M3, M2 and M4). If the purpose is callogenesis or rhizogenesis for bioactive substances extracts or other purposes, the culture on M14, M15 or M16 media is the most suitable.

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