Isolated culture of *A. reptance* L., its' morphological and growth features

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Abstract. A growing demand for the ecologically pure products brings us for searching novel biotechnological approaches for plant cultivation. One of these approaches is the *in vitro* cultivation and further acclimatization of valuable plant species. The object of our investigation was *Ajugareptance* L. ornamental plant which possesses high metabolic activity. *In vitro* cultivation was carried out applying Murashige-Skoog nutrient medium and its modifications. Acclimatization of *in vitro* plants was implemented according Hazarika. In the presence of twice higher concentration of cytokinins over auxins and 0.2 mg/ml gibberellins callus culture was formed from the leaf explants. Callus tissue was formed in the presence of 0.2 mg/ml kinetin and 2 mg/ml indole-3-acetic acid which has denser structure than the first one. The shoot formation was observed on callus cultures growing on the same medium approximately after 5^{th} passage. Callus culture growth was supported also by the adding of 2 mg/ml 2,4-dichlorophenoxyacetic acid. For the micropropagation, the already formed shoots were transferred to the nutrient medium which contains only 0.1 mg/ml 1-Naphthaleneacetic acid as a phytohormone. *A. reptans* culture has high regenerative ability and the micro-propagation index was $10^4 – 10^5$. In vitro regenerated plants were successfully acclimatized to the soil conditions during two-week period.
Keywords: *Ajugareptance L., in vitro cultivation, callus culture*

1 Introduction

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In vitro propagation is considered as an effective tool of obtaining rapidly a large number of plants, and for *ex situ* conservation of plants which are included in the lists of scarce or endangered species. Plant *in vitro*culture technique had been used previously with success for many endangered or economically valuable plants [1 – 7]. So, in some special cases it can be applicable to replace the traditional methods of cultivation.

Plants belonging to the Ajuga genus,in addition to their ornamental role, possess high metabolic activity [8 -10].Some species of this genus are categorized as endangered [10]. Many species of this genus produce substances with cytotoxic, insecticidal, antibacterial, antiviral, antioxidant agents [10 -12]. There are 4 species of this genus represented in Armenian flora (*Ajugachamaesistus* Ging. Ex Benth., *A chia* Schreb., *A genevenis* L., and *A. orientalis* L.) [13].

A.genevensis contains iridoids, flavonoids, glycosides, terpenoides, and steroids, possess anti-inflammatory, hemostatic, wound healing properties [9, 10]. *A chia* produce iridoids, alkaloids, flavonoids, triterpenoids, ursolic acid, fatty acids (palmitic acid, stearic acid, etc.), phytoecditeroids (cyasterone, exdisterone, turkesterone) and it is useful in folk medicine [14].

There is scarce information on Ajuga genus plant isolated culture. There are severalreports concerning some species of this genus (*A. reptans*, *A. genevensis*, *A. bracteosa*,*A. chia* and some others*)*, their cell and tissue cultures [8, 15 – 17]. Among others*A. reptance*can be of interest as it is known to have wide spectrum of biological activity and used for medicinal purposes since ancient times [18]. Many researchers state that *A. reptance* is characterized by the presence of 7 basic ecdysteroids (ayugalactone, cyasterone, sengosterone, 29-norsengosterone, 29-norcyasterone, 20E and polypodine B) [18-20]. These substances are produced in different quantities depending on the cultivation conditions. The authors note that ecdysteroids synthesize in roots of this culture [21]. Previous studies of the same author have shown that the production of exosteroids in *A. reptans* culture depends also on the level of culture differentiation and calluses derived either from the leaves or roots are not able to synthesize exdosteroids[22, 23].In other reports it is mentioned that *A. reptans* isolated culture can serve a source of ecdysteroids but there occur a tendency to decrease the synthesis of these substances in parallel to increase of cultivation period[20].

As we see the literature data about this plant isolated culture biological activity are sometimes mutually exclusive. So additional data is in need in order to systematize the existing available information and give a general conclusion. The first step in this work will be obtaining *in vitro* culture of this plant for further investigation its' metabolomic characteristics and their possible application area.

A. reptance is not native for Armenian flora and there is notany data of its' occurrence as invasive plant for this particular region [13]. So, the micropropagation and *in vitro* cultivation of this species can be of interest, taking into account its high ornamental value as well as property to synthesize medicinally valuable metabolites.

The aim of the presented work is to develop the conditions for obtaining *A. reptans* isolated culture, to study its growth and some metabolic features.

2 Materials and methods

2.1 Donor Plant Material

A. reptans plants were purchased from the local market (Yerevan, Armenia). Seedlings were transferred to the soil and leafs were used as explants for further investigations.

2.2. Cultures and Nutrient Media

Callus cultures of leaf origin were obtained by sterilizing with mixed solution of cetylpyridinium chloride (660 mg/L) and mercuric chloride (330 mg/L) for 7 min. Murashige - Skoog (MS) [24] nutrient medium (with addition of glycine (2.0 mg/L), indole-3-acetic acid (IAA) (2.0 mg/L) and kinetine (6-furfurylaminopurine) (0.2 mg/L)) was used. The Petri dishes with explants (approx. diameters of leaf-origin explants were 0.8 to 1.2 cm, root origin – 1.0 cm in length) were placed in thermostat under the thermal conditions of 22- 25° C (for the initiation of proliferation processes). Afterwards the formed primary callus tissues were placed in the flasks (50 mL), and then replaced in thermostat (22-25° C, in dark conditions).Shoot proliferation was took place in callus cultures on the same nutrient medium. Callus growth was supported also using MS basal media with addition of: a) gibberellic acid (GA) (0.2 mg/l), 1-naphthaleneacetic acid (NAA) (0.5 mg/l), kinetine (1 mg/l) and 6-benzylaminopurine (1 mg/l), which was conditionally named medium N7; b) IAA (2.0 mg/L), kinetine (0.2 mg/L), and 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/ml). The medium both without cytokinins and with (NAA) (0.1 mg/L) and sucrose (20 g/L) was used for the root origination (medium R). Callus cultures of root origin were obtained from the formed roots of *in vitro* plantlets without sterilization. All of the *in vitro* plantlets were incubated at 22-25° C with the photoperiod of 16 h (natural daylight, supplemented with artificial light (approx. 150 μmol quanta m^{-2} s^{-1}) provided by a white fluorescent lamp (Philips Inc., 36 W). The pH of the all culture media was 5.8 before adding agar and media were sterilized by autoclaving at 120° C for 20 min.

2.3 *A. reptance* culture cytomorphology

For the cytomorphological investigations tiny slides were prepared from the callus tissues with further staining by safranin.

2.4 *A. reptance* micropropagation parameters

During the proliferation stage, four subcultures were done at intervals of four weeks, to the same proliferation medium, and the cultures were maintained in the growth room under the same conditions of illumination and temperature used during the introduction stage. After the final cultivation cycle, the shoots were individualized and the number of shoots per initial explant in each treatment was counted.

2.5 Growth parameters of *A. reptance* callus cultures

Plant tissues were dried to constant weight at 60 ºC. Dry weight (DW) of tissues was calculated at the end of every five days up to 30 days by the formula giving below :

$$DW\ (\%) = Final\ dry\ weight\ of\ tissue/Fresh\ weight\ of\ tissue \times 100 \quad (1)$$

Growth Index (GI) of callus tissue was calculated at the same terms by the following formula:

$$GI = Final\ dry\ weight\ of\ tissue - Initial\ dry\ weight\ of\ tissue/\ Initial\ dry\ weight\ of\ tissue \quad (2)$$

2.6 Acclimatization

Plantlets that were observed to have well-developed roots and shoots (up to 5 - 6 cm) after four weeks were transferred to a containers filled with sterile substrate and kept covered with transparent waterproof cover for 7-10 days providing regular air access. Afterward the transparent cover was removed and plantlets were acclimatized to the greenhouse conditions, followed to the acclimatization to the soil conditions. The survival rate was calculated after one month [25].

2.7 Data processing

Experimental data were expressed as the mean ± SD of all repetitions. Values were normalized to the control and are given as average of the repetitions. A statistical analysis was done with the Student-t test for calculating the probability values; and data were considered statistically different at a *p*-value of 0.05 or less.

3 Results and discussion

Callus culture formed on *A. reptance* leaf explants both on MS and N7 nutrient media (see Materials and Methods). Which were further used for the supporting of tissue growth during the 5-6 re-cultivations. Both of the used nutrient media induce growing of a tissue culture with dense structure, but medium N7, with an approximately four-fold increased concentration of cytokinins over auxins and containing GA, brings to the formation of callus culture with a less pigmentation and an ability to form spontaneous morphogenesis, meanwhile, culture, growing on the MS medium did not show any potential to promote active morphogenetic response (Fig. 1).

This was surprising as literature data suggest that during the reprogramming of gene expression as well as existence of some crucial changes in cell morphology, physiology and metabolism in the somatic cells the high level of endogenous auxin is considered as one of the central signals determining embryogenic competence [26] and in most cases the reduction ofexogenous auxin level is needed for the initiation of differentiation processes [27].

The obtained callus tissues were differing also by their growth parameters - accumulation of dry substances and growing indexes. The results of our investigations showed that N7 nutrient medium was effective also by the means of callus culture growth as there was a great difference (approximately five-fold) between the dry weights (%) and GIs of callus cultures (Fig. 2).

As it is mentioned above, for the callus culture growth it was applied also a nutrient medium, containing 2,4-D in a concentration of 1 mg/ml.This was carried out in order to suppress the highly exposed morphogenetic potential. This was necessary to maintain the growth of dedifferentiated tissue. Cultures on this medium were cultivated during 3 sequential re-cultivation procedures, after which they completely loosed their ability to express morphogenetic potential (data not shown). The further cultivation of these tissues on N7 nutrient medium brought to the recovery of the morphogenetic ability.

Embryogenic callus was formed on leaf explants cultivated in dark conditions on N7 nutrient medium. Embryos at this stage were observed for 8-10 days, and then they were replaced under the light conditions for 20-25 days in order to promote shoot formation. In this period of cultivation it was not observed any formation of suspensor-like structures (data not shown), which is evidence of multi-cellular origin of formed embryos [28]. This phenomenon has also been described in other sources in the literature [26].

Fig. 3. presents the main phases of embryogenesis where it was observed the formation of procambium (pc), surrounding by the meristem (gm) in the different locations of callus tissue (Fig. 3). A mutually inhibitory action between auxin and cytokinin signaling is an important aspect of procambium formation [29]. Procambial cells have propensity to form root meristems or vascular tissues in response to added auxin [30].

Histological observations were revealed the existence of embryogenic zones also when applying the nutrient medium with low hormone concentrations (see Materials and Methods). Plant regeneration evidences wererevealed on 50–70% of calli. An elevated capacity to form somatic embryos and regeneration on N7 nutrient medium was maintained during the long-lasting cultivation (for more than 2 years).

Fig. 1. *A. reptance* callus cultures, cultivated on N7 (a) and MS (b) nutrient media (scale bar is 1 cm).

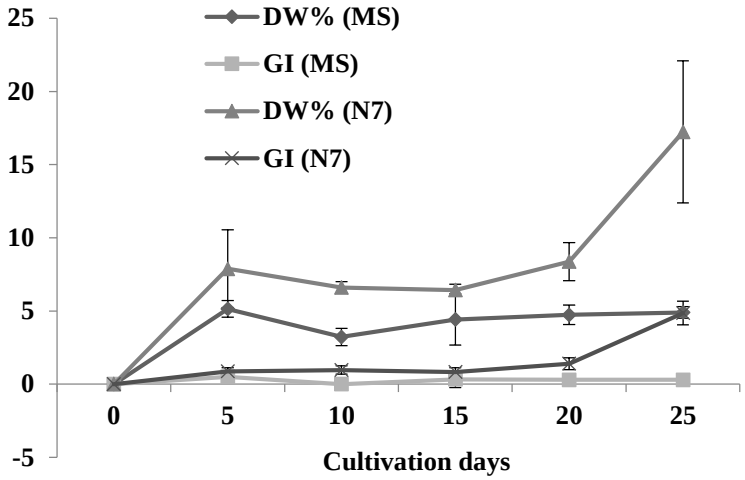

Fig. 2. Growth kinetic curves of *A. reptance* callus cultures, cultivated in MS and N7 nutrient media. DW%- dry weight, GI - growth Index.

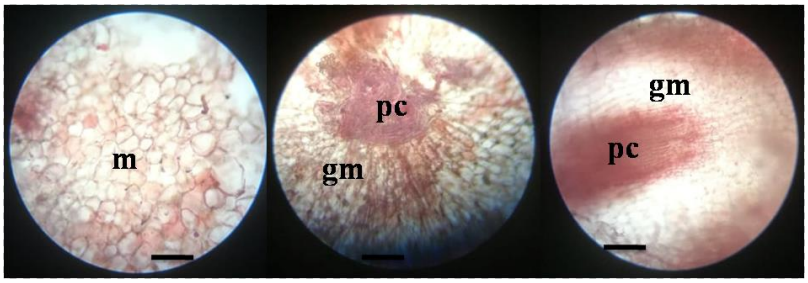

Fig. 3. Secondary differentiation in *A. reptance*callus cultures, growing on N7 nutrient medium (all scale bars are 50 μm): m – meristem, gm – groung meristem, pc - procambium.

The number of regenerated shoots depended on the explants size and medium composition. Interestingly, in parallel to the shoot formation the intensive formation of roots was also observed (Fig. 4 (a)). After two-week cultivation the new-formed plantlets were transferred to the regeneration medium (R), where they effectively continued their growth and multiplied shoots and roots (Fig. 4 (b)). Long-term (2 – 2.5 years) cultivation had no any visible influence on *A. reptance* isolated culture growth features (Fig. 4). The plant formation process from the somatic embryos was lasted approximately 20-25 days.

It was clear that*A. reptance* isolated culture possessed high-frequency regeneration ability (index of micro-propagation was 10^4 -10^5). Our previous investigations data showed that in some cases plants obtained from the plant of the Ajuga genus reached blooming phase during *in vitro* cultivation during May-June period which corresponded to the natural cycle of development [9]. In this particular case it was not observed any initiation of blooming, meanwhile, 90-100% ($p<0.05$) of obtained *in vitro* plantlets was successfully acclimatized in a soil substrate during two-week period (at the stage when they reached 3-5 cm high) (Fig. 4 (d, e)).

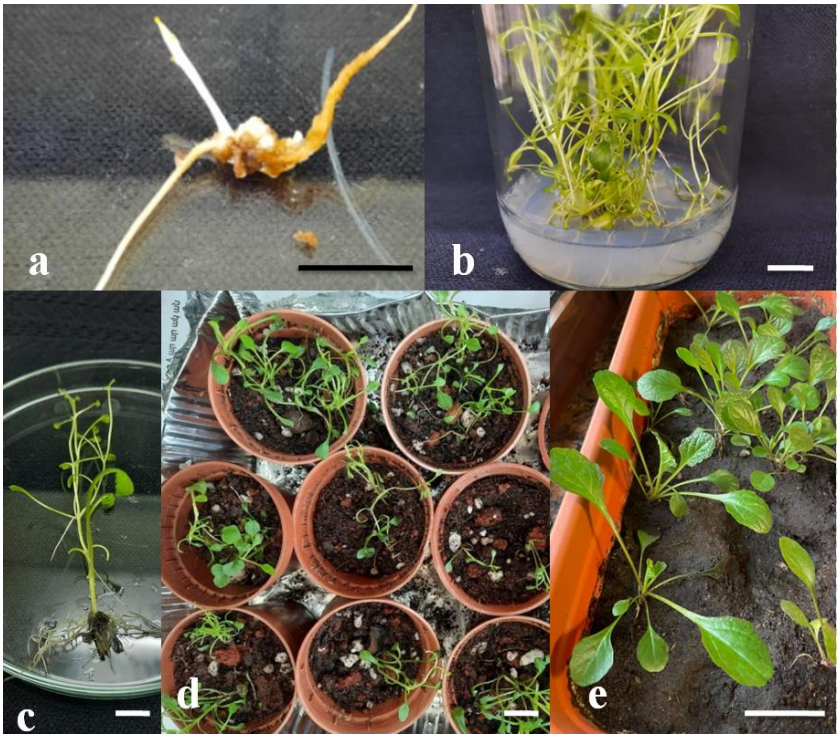

Fig.4. Somatic embryogenesis in callus culture of *A. reptans*, cultivated on the N7 nutrient medium: a – shoot and root formation on callus tissue (scale bar is 1 cm); b – Plantlets, growing on the R medium (scale bar is 1 cm); c – plantlet with a developed shoots and root system, ready to acclimatization procedure (scale bar is 1 cm); d, e –plants on the different stages of acclimatization (scale bars are 1 and 5 cm, respectively).

4 Conclusion

The phytohormonal composition of both N7 and MS nutrient media supported callus formation. Differences in content of phytohormones in applied mutrient media influence the expression of culture's regeneration potential: approximately after 5 cycles of periodic cultivations a spontaneous embryogenesis was occurred on the medium, where auxin concentration was four-fold higher over cytokinins.This observation results can be applicable for cultivation of plant with remarkable medicinal, agricultural and ornamental values.

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