Adventitous Bulblet Regeneration Of Endemic Ovacik Garlic
(*Allium tuncelianum* Kollman, Özhatay, Mathew, Şiraneci) Using
Wintered Half Clove Explant

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Abstract
Ovacik garlic (*Allium tuncelianum* Kollman, Özhatay, Mathew, Şiraneci) is an important endemic and threatened plant species of Eastern Turkish province of Tunceli and its surrounding areas with poor seed set and germination problems. There is an urgent need to develop strategies for protection and conservation of the plant by the development of in vitro conservation protocols. Hence, the present study was focused to develop an efficient regeneration protocol of wintered and unwintered half cloves of the Ovacik garlic that were cultured on 0.25, 0.50 and 1.0 mg L⁻¹ Benzylaminopurine (BA) with 0.25, 0.50 and 1.0 mg L⁻¹ of Potassium salt of Naphthaleneacetic acid (KNAA) for regeneration. Unwintered upper and lower half clove explants failed to regenerate new bulblets. Similarly, no regeneration was recorded on wintered upper half of the Ovacik garlic. Proximal half clove of the wintered Ovacik garlic was evaluated as best explant for regeneration on MS medium containing 0.50 mg L⁻¹ BA with 0.50 mg L⁻¹ KNAA. Regenerated bulblets were rooted on MS medium supplemented with 60 g L⁻¹ sucrose. The rooted bulbs were acclimatized and transferred to pots and fields. It was concluded that the protocol could be safely used to conserve this plant.

Keywords: Adventitous, Clove, Endemic, Garlic, Medicinal

1. Introduction
The genus *Allium* of Alliaceae family has a diverse taxon comprising of nearly 500 species and 164 of these originate in Turkey. Forty percent of *Allium* species found in Turkey are endemic [1]. Ovacik garlic or Tunceli garlic (*Allium tuncelianum* Kollman, Özhatay, Mathew, Şiraneci) [2] is endemic to plateau surrounding Munzur Mountains in Ovacik, Hozat, Tunceli and Plumur districts of the Eastern Turkish province of Tunceli and regions located between Sivas and Erzurum provinces [3-4]. Ovacik garlic and *Allium longicuspis* are considered wild ancestor of modern garlic [5]. Ovacik garlic is a single cloved cream white bulb with non-bulbiferous inflorescences and white flowers with fertile black seeds.

Ovacik garlic is extensively used as an alternate to garlic for making food in the Tunceli province of Turkey and its surroundings. Approximately, 15-25 ton of Ovacik garlic has been collected from wild [6] for domestic use and for medicinal purpose [5] by herbalists in Turkey. Ovacik garlic contains 37.94 % dry matter and 0.08 µg Allisin and 0.36 µg Alliin. It stimulates the body's immune system, lowers the level of cholesterol and sugar in the blood. It dilutes the blood and improve blood circulation thus reduces the chance of heart attack [7].

Collection of endangered geophytes for trade is banned in Turkey for conservation purpose in agreement with Convention on the International Trade in Endangered Species (CITES) [8-11]. However, over-exploitation of the plant associated with poor seed set and germination has
made it an endangered species [12] with threat to extinction. There is dire need to develop strategies to conserve Ovacik garlic before its extinction. Conservation efforts can be complimented by development of in vitro conservation protocols along with improved agronomic techniques [13] suitable for cultivation of the plant in other areas of Turkey.

Application of plant cell and tissue culture techniques for vulnerably threatened and endemic Ovacik garlic has lagged behind when compared to other garlic species. Although, efforts has been done to regenerate plants under in vitro conditions [3, 12]. But still, there is need for doing extensive work for development of comparatively more efficient and reproducible tissue culture protocols. The present study was designed with objective to optimize conditions for bulblet regeneration, rooting, hardening and acclimatization to increase the efforts for Ovacik garlic conservation.

2. Material and Methods

Medium sized Ovacik garlic bulbs (2.5 - 3 cm diameter) were obtained from Prof. Dr. Suleyman Kizil, the Department of Field Crops, Dicle University, Diyarbakir, Turkey. They were washed under tap water using detergent followed by drying on filter papers. Thereafter, they were divided into two lots. One lot of Ovacik garlic cloves were subjected to surface sterilization with 100% commercial bleach (ACE, Turkey containing 5% NaOCl) for 10 min followed by 3 × 5 min rinsing with bidistilled sterilized water. The Ovacik garlic cloves of the other lot were wintered for four months under clean, cool and dry place. Thereafter, they were surface sterilized as described above.

![Figure 1. Anatomy of clove of Ovacik garlic bulb/clove with distal and proximal halves used as explants.](image)

The bulbs were cultured on MS medium [14] supplemented with 3.0 % sucrose gelled with 0.65% agar (Duchefa) for 15 days for selection against fungus and bacteria. Thereafter, the surface sterilized Ovacik garlic bulbs were horizontally sectioned into distal and proximal halves such that the upper part of the clove contained fleshy clove specialized leaf sheath, upper part of sprout leaf, cavity and onion tip. Whereas, the proximal half of the clove contained basal plate, fleshy clove with specialized leaf sheath and lower part of the sprout leaf (Fig. 1).

The explants were cultured on MS medium supplemented with 0.25, 0.50, 1 mg L⁻¹ BA with 0, 0.25, 0.50 and 1.00 mg L⁻¹ KNAA, 3.0 % sucrose and gelled with 0.65% agar. The explants were sub-cultured after every four weeks. The first data were taken after eight weeks and the subsequent data were taken after 16 weeks of culture. Thereafter, the regenerated bulblets were isolated from the explants carefully and transferred to MS medium devoid of plant growth regulators containing 60 g L⁻¹ sucrose for rooting. After 16 weeks of culture, the rooted bulblets were transferred to pots containing coarse grained sand and organic matter (1:1). The bulblets in pots were initially grown under controlled environmental conditions for 10 days in the growth chamber to start their photosynthetic system functioning...
necessary for acclimatization. Thereafter, the plants were transferred to greenhouse and subsequently to fields.

The pH of all culture and rooting media were adjusted to 5.6 - 5.8 using 0.1 N KOH or 0.1 N HCl before autoclaving at 118 kPa and 121°C for 20 minutes. All cultures were incubated in growth chamber (Sanyo E&E Europe BV, Biomedical Division, UK) at 20 ± 1°C with 16 h light photoperiod.

The experimental design was one factorial and involved twelve treatments with 9 replications each containing five explants (4 explants x 9 replications = 36 explants/treatment) excluding control containing MS medium. Data for frequency (%) of bulblet regeneration, mean number of bulblets per explant, bulblet diameter and frequency (%) of rooting was subjected to one way ANOVA using F test with computer statistical software IBM SPSS statistics 20 for Windows. The post hoc tests were performed using Duncans Multiple Range Test (DMRT) to compare the differences among control and treatments. Data given in percentages were subjected to arcsine square root transformation [15] before statistical analysis.

3. Results

3.1. Shoot regeneration from unwintered explants. No regeneration was recorded on MS medium from unwintered upper and lower half clove explants (Table I). The results showed that lower and upper halves of the clove taken from unwintered bulbs used for in vitro culture was not suitable for regeneration. The upper halves of the unwintered bulb explants increased in size. The leaf sheath in the center grew and pierced out from the bulb tips on the dorsal side to about 1 cm and on the ventral side of explant to about 0.5 cm (Fig. 2a). Similarly, the lower halves of unwintered bulbs changed from cream white color to green and increased in size such that visible green colored single or cylindrical stalk of superimposed tightly wrapped sprout leaves pierced out on dorsal surface of the explants in the central portion. Rooting was also recorded from basal plate on ventral side of the explants; however, no adventitious or axillary bulblet regeneration was noted on explants even after 16 weeks of culture (Fig. 2b).

3.2. Shoot regeneration from Wintered Explants. The increase in explant size was almost negligible on upper half cloves. Similarly, change in color of fleshy leaf sheath was also not very visible. However, the sprout leaves grew 1-2 cm and pierced out of the clove tips. No regeneration was recorded on these explants after 16 weeks of culture. No bulblet or callus induction was recorded when the upper half clove explants were cultured on MS medium containing either 0.25, 0.50 or 1 mg L⁻¹ BA used singly or with any concentration of KNAA. On the other hand, lower half clove explant showed response to growth variants. However, an analysis of the results showed that MS medium containing any concentration of BA singly was not sufficient to induce bulblet regeneration. Similarly, 1 mg L⁻¹ BA with any concentration of KNAA failed to induce regeneration. The basal plate on ventral side was not directly involved in bulblet induction. However, removal of basal plate promoted the separation of sprout leaf/leaves from fleshy clove that ultimately resulted in single shoot development and were not accounted for data taken. The lower half clove/bulb explants induced variable number of leaf meristems on ventral surface of the explant after 20 days of culture. After four weeks of culture, the meristems developed into visible leaves (Fig. 3a) followed by induction of cream white or off white colored bulb-like swellings (Fig. 3b) at the proximal ends without any callus induction, which converted to bulblets after 4-5 weeks of culture. An analysis of culture media containing 0.25, 0.5 and 1 mg L⁻¹ BA with variants of KNAA after eight weeks of culture further indicated that the bulblet regeneration ranged 0-75% with 0-5.33 bulbs/explants and
bulb diameter range of 0.39 to 0.45 cm. Maximum bulblet regeneration (75.0%), bulbs/explants (5.33) was recorded on 0.50 mg L⁻¹ BA-0.50 mg L⁻¹ KNAA (Table I).

The experiment was run for further eight weeks to check its effects on regeneration behavior. No increase in frequency or mean number of bulblets per explant was recorded after 16 weeks of culture. However, positive increase in the bulblet diameter was very evident (Fig. 3c, d). The new increase in bulblet diameter ranged 0.82 – 0.98 cm for 0.25 mg L⁻¹ BA with variants of KNAA and 0.62 -0.67 cm for 0.50 mg L⁻¹ BA with variants of KNAA (Table I). Maximum increase of 0.55 cm in bulblet diameter was recorded on MS medium containing 0.25 mg L⁻¹ BA-0.50 mg L⁻¹ KNAA (TABLE I). The results further indicated that MS medium containing 0.25 mg L⁻¹ BA with 0.50 mg L⁻¹ KNAA regenerated largest bulbs compared to the bulbs regenerated on MS medium containing any other variant of BA - KNAA.

3.3. Rooting and Acclimatization

After 16 weeks of culture, regenerated bulblets were carefully separated from cloves and transferred to MS medium supplemented with 60 g L⁻¹ sucrose with no auxins. 100 % rooting along with increased bulb size was recorded after 16 weeks of culture. Rooted bulblets were transferred to pots containing sand and organic matter (1:1) and kept under controlled environmental conditions in growth chambers for 10 days that helped to face the problems during early development and hardening. It was very effective and the bulblets began to grow in size with visible growth of foliage and sprout leaves on the bulblets. Thereafter, the pots were transferred to growth room for 6 weeks followed by transfer to field conditions. All plants subjected to acclimatization were not difficult to establish both in pots and in the fields.

4. Discussion

Classical propagation methods of Ovacik or Tunceli garlic have been carried out by bulbs, bulbils, bulblets and seed. However, the propagation rate and number of plantlets produced are not so high for practical propagation [12]. This study presents successful adventitious bulblet regeneration, rooting and acclimatization of Ovacik garlic that is an important threatened medicinal endemic plant of Turkey using proximal half of wintered bulbs/cloves as explant.

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<th>Table I</th>
<th>Effects of BA and KNAA on adventitious bulblet regeneration of endemic Ovacik garlic</th>
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Mean values within a column followed by different letters are significantly different at 0.05 probability level using Duncan’s multiple range test.
No regeneration could be induced on upper half cloves of both wintered and unwintered explants on MS medium (control) or MS medium containing any concentration or combination of plant growth regulators. Similarly, no regeneration was recorded on upper half explants obtained from wintered bulblets except development of foliage leaves. Contrarily, the wintered proximal half clove responded well to the variable concentrations of plant growth regulator combinations in the culture medium. The difference in response of upper and proximal half wintered clove explants might be due to the structural variations of the explants.

The results further showed that BA alone in the culture media is not sufficient to induce bulblet regeneration. BA concentrations could only induce regeneration in the presence of potassium salt of NAA (KNAA). It is assumed that Potassium, which is required for cell growth of plant species served as an activator of enzymes used in photosynthesis and respiration and helped in building of cellulose and aided in photosynthesis by the formation of a chlorophyll precursor [16] and thus helped in formation of bulblets and their easy establishment.

Results further showed that explants type was another important factor for efficient bulbs regeneration along with winter treatment and KNAA. The presence of basal plate at proximal half clove explants did not directly induced the bulbs regeneration but certainly enhanced the multiple adventitious bulbs regeneration. Micropropagation of Tunceli garlic from root tips has also been reported by Kizil & al. (17). The basal plates were used as explants for in vitro tissue culture of garlic, for establishment of somatic embryogenesis and plant regeneration by XUE & al. [18] and KOCH & al. [19], who reported improved regeneration of shoots from garlic callus induced from basal plate culture. Similarly, AASIM & al. [20] also reported bulblet regeneration from basal plate of twin scale explants of red squil.
Results further showed that combination of growth variants (BA and KNAA) is important for bulblet regeneration. The results were further supported by the findings that any concentration of BA singly failed to induce bulbs and maximum bulbs regeneration and bulbs/explant were recorded from equal concentration of both variants (0.50 mg L\(^{-1}\)) in the culture medium. Contrarily, YANMAZ & al. [12] failed to induce shoots form bulblets placed in both IAA and BA combinations. Furthermore, they found that IAA application gave the best bulblet regeneration after fourth subculture with poor quality of bulblets. They also noted that bulblet initiation started after second subculture in cytokinin free medium containing 0.1 mg L\(^{-1}\) NAA. Variations in results might be due to selection of explants at different stages of biological growth, use of different plant growth regulators or variable combinations of plant growth regulators used in the two experiments. The results also confirmed that the equivalent concentrations in terms of mg L\(^{-1}\) of BA and KNAA in the culture medium were important for maximum bulblet size.

The ability of auxins to promote rooting of ornamental plants has been known since long; however, many plant species do not need auxin applications for rooting. All regenerated bulblets rooted with increased in diameter on MS medium devoid of auxins containing 60 g L\(^{-1}\) sucrose after 16 weeks. The rooting of Ovacik garlic without auxin treatments could be compared with GADEL-HAK & al. [21], who found that both shoot and roots were induced on MS medium without growth regulators. Similarly, CAMARA & al. [22] indicated that garlic rooting could be achieved without auxins on MS medium. The results are also in agreement with the rooting from other important bulbous geophytes like *Ornithogalum ulophyllum* [10] and *Muscari macrocarpum* [23]. However, YANMAZ & al. [12] obtained the highest rooting ratio of 17% and 33% on Tunceli garlic cultured on 0.5 mg L\(^{-1}\) and 2.0 mg L\(^{-1}\) NAA treatment respectively. Rooted bulbs were successfully acclimatized in pots under greenhouse conditions and under field conditions. Whereas, *in vitro* rooted and unrooted regenerated Ovacik garlic bulblets died after second or third leaves were generated [12].

**Conclusion**

Poor seed set, combined with ruthless collection of the plant by locals for local markets using destructive harvesting techniques and habitat destruction in the form of deforestation has added to the magnitude of the problem. The present study, established efficient protocols for in vitro propagation of clonally uniform plants of Ovacik garlic and provides a view of biotechnology that can contribute to the conservation process and development of disease free, true-to-type superior quality bulbs. Therefore, there is need to develop more tissue culture protocols using other explants for utilization in breeding, propagation and conservation of Ovacik garlic.

**References**

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