



Using different phytohormones and putrescine as treatment boosters to induce regeneration from endospermic immature embryos of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci

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Abstract

Tunceli garlic (*Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci) is an economically important endemic and rare plant species spread among Tunceli, (Hozat, Ovacık, and Pulumur counties), Sivas, and Erzincan provinces in Turkey. The primary goal of the study was to devise ways to induce and improve plant regeneration from Tunceli garlic immature zygotic embryos. The viability of tested seeds was confirmed through the tetrazolium test. Thereafter regeneration was further tested through culture on water agar, several concentrations of (i) 2,4-dichlorophenoxyacetic acid (2,4-D), and (ii) 6-benzylaminopurine (BAP), potassium salt- α -naphthaleneacetic acid (K-NAA), and 300.0 mg L⁻¹ putrescine used as an elicitor or regeneration booster. These results also confirmed that seed viability level varied during growth showing nonsynchronous seed maturity in the umbels. 2,4-D induced a maximum of 20% somatic embryogenic callus using 0.3 and 1.2 mg L⁻¹ 2,4-D treatments. Paradoxically, 6.66 to 13.33% bulblet induction was noted on BAP treatments. Any concentration of BAP and K-NAA or BAP and putrescine was also suppressive and showed a reduced regeneration of 6.66%. BAP, K-NAA, and putrescine combinations were synergetic and induced regeneration potential of 10.33 and 20% bulblets per explant. The results underscore that the putrescine elicitor triggers bulblet regeneration in association with BAP and K-NAA. Putrescine failed to induce bulblet regeneration when used singly with BAP. These results have high significance and will contribute positively to understanding the regeneration of *A. tuncelianum*.

Keywords *Allium tuncelianum* · Immature embryos · Micropropagation · Putrescine · Tunceli garlic

Introduction

Allium tuncelianum (Kollmann) Özhatay, B. Mathew and Şiraneci is an endemic single creamy off-white light yellowish colored, rarely two-cloved garlic species with 2n=16 chromosomes (Hanelt 1990; Özhatay 2002). It naturally grows and concentrates under oak forests in an area that extends from Hozat, Ovacık, and Pulumur counties of Tunceli province to adjacent foothills of Munzur Mountains

extending to Erzincan province on one side, and Sivas provinces on the other of Turkey (Fig. 1; Koyuncu and Guvenc 1994; Firat 2015).

More than 175 *Allium* taxa (Tubives 2024) are described in the *Flora of Turkey* (Davis 1988; Tubives 2024). There are reports of their usage locally in the ethnomedicinal systems of Turkey (Dal 2021).

Kollman (1983) identified two subspecies of *Allium* namely *A. macrochaetum* sub-species *macrochaetum* (distributed in Turkey and Syria) and *A. macrochaetum* sub-species *tuncelianum* (endemic to Turkey). Later taxonomic identification of the two subspecies was revised (Özhatay *et al.* 1997) and *A. tuncelianum* was accepted as a new species (Worldfloraonline 2024).

The spatial arrangements of organosulfur compounds in *A. tuncelianum* are very similar to *A. sativum*. The garlic taste comes from the breakdown of thiosulfate (Iberl 1990; Ferary and Auger 1996; Keusgen *et al.* 2002) alliin (2-propene-1-sulfinothioic acid S-2-propenyl ester), γ

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glutamyl-S-alkylcysteines, and cysteine sulfoxide peptides to sulfites (Lancaster 1988; Ferary and Auger 1996), which are antitumor (Karaaslan *et al.* 2019), antimutagenic, anticarcinogenic, antithrombotic, and antineoplastic, and have antioxidant properties (Thomson and Ali 2003; Nouroz *et al.* 2015; Farhat *et al.* 2021). Furthermore, *A. tuncelianum* is also used as a functional food. The experimental and epidemiological evidence also relates garlic consumption to treating cardiovascular diseases (Cavagnaro *et al.* 2007; Bradley *et al.* 2016) and cancer care (Nouroz *et al.* 2015).

It could also be used for ornamental purposes due to attractive lilac-colored florets in its umbels, growing on 25 to 60 cm long and wide stalks. The florets contain three-cornered, 1 to 2 mm diameter, seeds, or capsule-like fruits. The umbel weight of *A. tuncelianum* can reach up to 10 to 80 g at maturity depending on the environmental conditions and health of the plants. They have an average fresh a clove width of 2.8 to 3.4 cm and a length of 2.7 to 3.5 cm,

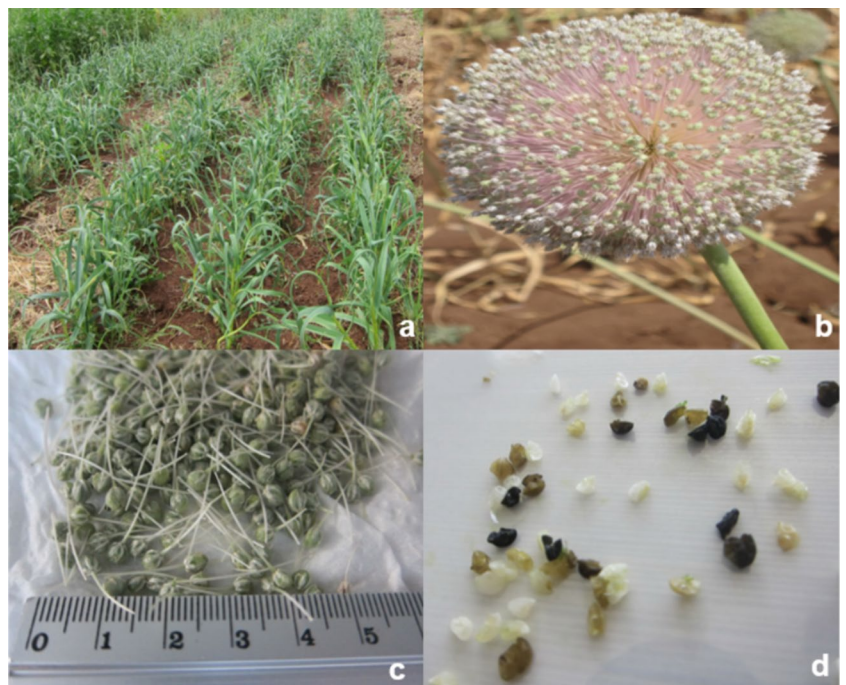
depending on the soil, environmental conditions, and the health of the plants (Ozhatay and Siraneci 1992).

This endemic species is under protection. It is included in the IUCN list of vulnerable (VU) plant species and has a mention in the *Red Book of Turkish Plants* (Ekim *et al.* 2000) and revised list (Tehditalindabıtkiler 2023; World-floraonline 2024). The adaptation experiments of Tunceli garlic are being carried out at various places in Turkey (Erden 2024). Based on the results of these experiments, the municipal authorities are encouraging its cultivation in the Tunceli province through incentives to the farmers since the 1980s. The results of field trials conducted in Tunceli and Diyarbakir provinces show that the plant can be cultivated successfully (Fig. 2a) (Taskin *et al.* 1997). The species bloom from early August to September. The inflorescence of *A. tuncelianum* occurs as a spherical umbel with a diameter range of 2 to 15 cm in umbel with 50 to > 400 tiny florets (Fig. 2b). The florets in an umbel

Figure 1. Main areas where *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci is grown under natural conditions at Tunceli province, Turkey (Tubives 2024; after modification).



Figure 2. *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci (a) growing in the field, (b) its umbel in early seed set stage, (c) collection of capsules with immature embryonic seeds after 15 d of anthesis, and (d) immature endospermic embryos with and without peeling seeds.



completely open in 1 to > 14 d and shed pollen before the stigma is fully receptive (protandry), which promotes outcrossing with limited self-pollination. Both pollination and viable seed set tendencies are unpredictable and plants mainly depend on insects or wind pollination. The seeds in the umbels do not mature uniformly and synchronously. There is a need to make three to four pickings to collect the matured seeds. This leads to a nonuniform seed set with physiological dormancy occurring in the seeds (personal observations, Prof. Dr. Süleyman Kizil-2024 — unpublished data). Similarly, Dal (2021) reported in his study in Şanlıurfa conditions that the flowering period of *A. tuncelianum* varied between 20 and 27 d depending on the bulb size. Khokhar (2014) has reported that the regeneration of inflorescence (umbel) of species in the genus *Allium* during the generative stage is under complex genetic and environmental regulatory control with an indeterminate habit of growth. Therefore, many seeds of *Allium* species are destroyed in the umbels due to physiological immaturity that does not allow a complete seed set (Kamenetsky 2007) and germination.

Conventional methods of sexual or vegetative multiplication of the species through cloves and seeds, or raising them in nurseries before sowing have many limitations. *In vitro*, shoot regeneration is a basic step in introducing biotechnological approaches for adventitious and axillary bulblet regeneration (Yasemin and Beruto 2024). Yazar (2006) studied Tunceli garlic propagation and found root tip culture, which failed to induce callus formation. Shoot tip culture with medium containing IAA and NAA-induced bulb formation. Again Yanmaz *et al.* (2010) reported shoot culture from roots using different combinations of lower doses of IAA, BA, and NAA with Murashige and Skoog (MS; Murashige and Skoog 1962) medium. Kizil *et al.* (2014) found that root tip explants were most suitable for bulblet regeneration on MS medium. Aasim (2015) used wintered and unwintered half cloves and induced bulblets. They found that the proximal half clove was the best explant for shoot regeneration. Kaska *et al.* (2018) observed gynogenic regeneration on buds of *A. tuncelianum* on cytokinin and auxin-modified MS medium improved gynogenesis and somatic embryogenesis. Similarly, polyamines like putrescine open important mechanisms for plant regeneration and act as a potential booster during plant morphogenesis under *in vitro* conditions to multiply cells and fasten regeneration (Flores and Galston 1984; Galston and Kaur-Sawhney 1990; Ziemmer *et al.* 2024). Developing new regeneration techniques will facilitate rapid multiplication and will play a significant role in developing the Tunceli garlic tissue culture industry. No previous study has reported putrescine as a treatment booster to induce regeneration from immature endospermic embryos of *A. tuncelianum*. Therefore, the current study aimed to find the potential effects of different phytohormones and

putrescine as booster to trigger bulblet regeneration from immature endospermic embryo explants of the endemic *A. tuncelianum*.

Materials and Methods

Plant Materials The immature endospermic embryos of the *A. tuncelianum* (Fig. 2a, b) were used as experimental materials in the research. They were obtained from The Bulbous Plants Collection Garden of the Department of Field Crops, Faculty of Agriculture, Dicle University, Diyarbakir, Turkey (15 d after anthesis) in the morning ((06:00 to 07:00) (Fig. 2c, d)). Since seed formation in an umbel is not synchronous, therefore, there is a possibility of collecting immature endospermic embryos of different ages.

Immature endospermic embryos were carefully placed into paper bags without causing any injury and transferred to the laboratory under a cold chain. Each capsule contains two seeds. The duration of flowering is about 23 to 25 d under field conditions.

Tetrazolium Test It was important to know the viability percentage of the immature embryonic embryos before experimenting for comparison. This information gave an idea about the viability of the seeds used for regeneration. Monitoring of the viability of immature endospermic embryos in the umbels was done with the cost-effective 1.0 mg L^{-1} TTC (2,3,5-triphenyl tetrazolium chloride) (Merck, Darmstadt, Germany) method, which is based on the ability of any live tissues to reduce TTC into the red compound formazan (Larcher 1969). Four lots of randomly selected 100 seeds each were tested to confirm viability.

Preparation and Sterilization of Explants Thirty umbels were collected from the field-cultured plants to collect the immature endospermic embryos in the seed capsules. Each umbel contained 300 to 400 capsules. These were peeled to collect immature endospermic embryos. These (100 seeds for each sterilization) were carefully washed with water before sterilization by keeping them under gently running tap water in a 100- μm sieve for 30 min, followed by treating them with 100% commercial bleach (5.0% sodium hypochlorite) (ACE, Turkey) for 20 min and rinsing for 3×5 min with double-distilled autoclaved water. The surface sterilization procedures of seeds (seed humidity (40 to 45%) were carried out under sterile laminar flow (Thermo Scientific, Langensfeld, Germany) conditions carefully without damaging them under aseptic conditions.

The capsules were cut open to take immature endospermic embryos embedded in endosperms and covered with seed coats for culturing them at $24 \pm 1^\circ\text{C}$, under a photoperiod of 16-h light ($45 \mu\text{m}^{-2} \text{ s}^{-1}$) and 8-h darkness, on water agar

(0.62% agar), or Murashige and Skoog (MS; Murashige and Skoog 1962) medium (INTERLABMerck, Darmstadt, Germany, used as control), or MS medium amended/modified with different concentrations of auxin and cytokinin, supplemented with 3.0% (w/v) sucrose (Duchefa Biochemie Haarlem, Netherlands) and solidified with 0.62% (w/v) agar (Duchefa Biochemie) in two experiments as shown below. Double-distilled water was used in the preparation of each culture.

Experiment I MS medium modified with several concentrations of 2,4-dichlorophenoxyacetic acid (2,4 D) (Merck, Darmstadt, Germany) using 0 (water agar as control treatment), 0.3, 0.5, 0.7, 0.9, or 1.2 mg L⁻¹ 2,4-D (Merck) (experiment 1). Thereafter, these somatic embryos were fixed for 11 to 12 h with formalin acetic acid-ethyl alcohol (Isolab Ethanol, Interlab, İstanbul, Turkey). Their dehydration was done with ethyl alcohol and a series of butanol (Isolab Ethanol, Interlab) followed by embedding in paraffin (Merck, Darmstadt, Germany), which was microtomed to 10-µm sections and stained with safranin and fast green (Yilmaz and Khawar 2024).

Experiment II MS medium modified with agar in water was used as control. Other treatments using MS medium modified with 0.1, 0.3, 0.5, 0.7, or 0.9 mg L⁻¹ 6-benzylaminopurine (BAP) (Merck) singly (Table 2) or five combinations of BAP and 0.5 mg L⁻¹ potassium salt-α-naphthaleneacetic acid (K-NAA) (Merck) (Table 3), five combinations of BAP and 300.0 mg L⁻¹ putrescine (Merck) (Table 4), and five combinations of BAP and 0.5 mg L⁻¹ K-NAA and 300.0 mg L⁻¹ putrescine (Table 5) were used as regeneration booster.

Culture Conditions After adjusting the pH of each culture in the MS medium, their pH was corrected to 5.6 to 5.8 using 1 N NaOH or 1 N HCl. The sterilization was achieved in an autoclave (Selecta, Barcelona, Spain) at 121 °C under 1.4 kg cm⁻² pressure for 20 min. All cultures were kept in climate cabinets (Fitotron SGC 120, Liecestershire, UK) under white, fluorescent light at 24 °C with a fixed photoperiod of 16 h of light (45 µ m⁻² s⁻¹) and 8 h of darkness (Kizil *et al.* 2014).

Statistical Analysis of the Experimental Data Statistical analysis of the data from each experiment was subjected to one-way ANOVA using the “IBM SPSS 20 for Windows” statistical software. This parametric test compares the means of > 3 or independent groups and determines if there exist significant differences through statistical evidence among population means. One-way ANOVA is a balanced design parametric test. These are further tested using a *post hoc* LSD test.

Normally distributed dependent variables were presented as mean ± standard deviation (SD). Regardless of the experimental details, the combinations of the phytohormones (water agar as a control treatment, several doses of 2,4-D single or different combinations of BAP, K-NAA, and 300.0 mg L⁻¹ putrescine as an elicitor or regeneration booster were tested BAP and putrescine, K-NAA and putrescine, along with BAP, K-NAA, and putrescine) were used in the study. Each experiment used 60 explants divided equally into six replications (Petri dishes— 100 × 10 mm). Each Petri dish contained 10 immature seed explants. No seed germination occurred on the control treatment of water agar (control treatment). The experimental data were subjected to one-way ANOVA in a complete block single factorial experimental design using an LSD test to compare the treatment means. The % values were converted to arc-sine transformation before statistical analysis (Snedecor and Cochran 1967).

Results

Tetrazolium Test The results of the tetrazolium test carried out in the current study showed an average viability of 19.5% on immature endospermic embryos (results not shown in tabulated form). No study has been reported about testing the viability of immature *A. tuncelianum* seeds using the tetrazolium test until the writing of this report. Kizil and Khavar (2024) reported physiologic seed dormancy from mature seeds of Tunceli garlic under *in vitro* conditions and were able to break it after vernalization (cold treatment) for 81 d.

Water Agar No seed germination was noted on water agar used as a control treatment in any experiment. The moisture level of immature seeds was very high at the time of seed harvest but the seed germination and regeneration from the embryos depended on the level of maturity of the zygotic embryos (Sripathy and Groot Steven 2023).

The study used immature seeds (15 d after anthesis) as the explants on water agar and MS medium supplemented with combinations of BAP and K-NAA with and without elicitor putrescine which were insufficient to germinate them in line with Kizil *et al.* (2014) ending up with no germination on water agar (control treatment) through the presence of possible inhibitors (not tested) that may have negatively influenced germination. However, this inactivity was overcome after their interaction with different plant growth regulators or their combinations “dependent manner” in the form of regeneration.

Treatment with 2,4-D Regardless of the concentration of 2,4-D, significant differences were noted after treatment of endospermic embryos with different concentrations of 2,4-D. The experimental results showed irregular, inconsistent, and hyperhydric somatic embryogenesis on the induced callus after 28 d (Table 1). Somatic embryogenesis was observed on explants treated with any concentration of 2,4-D ranging from 6.66 ± 0.01 to $20 \pm 0.02\%$ except 0.5 mg L^{-1} 2,4-D.

The somatic embryos were converted to healthy plantlets by transferring to MS medium supplemented with 30.0 g L^{-1} sucrose. These somatic embryos could be easily identified at their globular and torpedo stages. All of them were in fused protoderm form. The conversion of somatic embryos to bulblets ranged from 1.22 to 12.30 (average count of 4.39 bulblets). The embryos induced on 0.3 mg L^{-1} 2,4-D could easily be separated (Fig. 3a) into individual plantlets. However, the embryos induced on other concentrations of explants were fused into groups of three, four, five, or more and were difficult to separate (Fig. 3b, c).

Use of 2,4-D has never been reported in tissue culture of *A. tuncelianum*. This treatment led to the expansion of the embryonic tissues; these tissues were surrounded by endosperm, formed of dome-shaped bodies, which differentiated into buds on each dome, to develop shoots and bulblets. These bodies appeared within 3 to 5 d of the culture

onset and independently developed somatic embryos 3 wk later, and were confirmed through histological observations. These observations showed that the internal cell organization and formation process of the dome-shaped structures were similar to those in the meristem. The formation of dome-shaped bodies appeared to be initiated by vigorous cell division in the epidermis of the tissue zones. Several studies have proven that this type of culture is useful for regeneration of virus-free plantlets.

Regeneration on MS Medium Amended with 5 Concentrations of BAP 2,4-D and BAP and NAA-based regeneration needed evidence to show if this is true regeneration instead of simply seed germination. Therefore, evidence such as figure images showing seed response on non-hormone medium vs BAP and NAA medium, and the timing of regeneration (being slower than germination shoots) were given for comparison purposes.

The results indicated reduced direct bulblet regeneration on BAP treatments with 6.66 ± 0.01 – $13.33 \pm 0.47\%$ regeneration, on MS medium modified with 0.1 to 0.9 mg L^{-1} BAP (Table 2). The minimum regeneration was noted on MS medium modified with 0.1 and 0.9 mg L^{-1} BAP. No regeneration was indicated on the MS medium modified with 0.3 mg L^{-1} BAP. Only 0.33 ± 0.01 and 4.00 ± 0.03 bulblets per explant (Fig. 4) were obtained on MS medium modified

Table 1. Effects of various concentrations of 2,4-dichlorophenoxyacetic acid (2,4 D) on callus regeneration percentage and number of somatic embryos regeneration per explant of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci immature seeds

2,4-D (mg L^{-1})	Callus regeneration (%)	Number of somatic embryos converted to plantlets per seed explant
0.3	$20.00 \pm 1.11\text{a}^{\dagger*}$	$12.30 \pm 0.69\text{a}$
0.5	$0.00 \pm 0.00\text{d}$	$0.00 \pm 0.00\text{e}$
0.7	$6.66 \pm 0.01\text{c}$	$1.22 \pm 0.11\text{d}$
0.9	$13.33 \pm 0.05\text{b}$	$3.11 \pm 0.02\text{c}$
1.2	$20.00 \pm 0.02\text{a}$	$5.34 \pm 0.01\text{b}$

*Different alphabetic letters represent statistically significant differences among 2,4-D treatments using LSD test at 0.05 level of significance; $\pm$ standard deviation

$^{\dagger}20.00 \pm 1.11\%$ is taken into consideration in measuring the callus regeneration or viability rate



Figure 3. Somatic embryo regeneration on *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci immature embryos (a) on Murashige and Skoog (MS) medium amended with 0.3 mg L^{-1}

2,4-dichlorophenoxyacetic acid (2,4-D), (b) fused seedlings with 0.9 mg L^{-1} 2,4-D, (c) fused seedlings recovered on MS medium amended with 0.3 mg L^{-1} 2,4-D. Bar = 0.85 cm.

Table 2. Average values of callus regeneration percentage, bulblet regeneration percentage, and number of bulblets per explant in *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci immature seeds using different concentrations of 6-benzylaminopurine (BAP)

BAP (mg L ⁻¹)	Callus regeneration (%)	Bulblet regeneration (%)	Number of bulblets per explant
0.1	6.66 c ± 0.01b*	6.66 ± 0.03c*	0.00 ± 0.00c
0.3	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00c
0.5	13.33 ± 0.35a†	13.33 ± 0.14a	4.00 ± 0.03a
0.7	13.33 ± 0.47a	13.33 ± 0.26a	0.00 ± 0.00c
0.9	6.66 ± 0.03b	6.66 ± 0.07b	0.33 ± 0.01b

*Different alphabetic letters represent statistically significant differences among BAP treatments using LSD test at 0.05 level of significance; ± standard deviation

†13.33 ± 0.35% is taken into consideration in measuring the callus regeneration or viability rate

**Figure 4.** Regeneration of bulb and shoots on immature embryos of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci on Murashige and Skoog medium containing 0.5 mg L⁻¹ 6-benzylaminopurine (BAP). Bar = 0.82 cm.

with 0.9 and 0.5 mg L⁻¹ BAP in the same order. The rest of the BAP concentrations failed to induce any bulblet.

Regeneration on MS Medium Amended with Five Concentrations of BAP and 0.5 mg L⁻¹ Potassium Salt of NAA (K-NAA) Treatments with modified MS medium with any concentration of BAP and 0.5 mg L⁻¹ K-NAA (Table 3) were further inhibitory compared to the results mentioned in the previous section. Callus induction (6.66 ± 0.09%) was noted on MS medium modified with 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA only. The rest of the combinations of BAP and K-NAA failed to induce callus. Direct bulblet regeneration percentage values of 6.66 ± 0.02%, 6.66 ± 0.01%, and 6.66 ± 0.03%, were noted on MS medium modified with 0.1, 0.3, and 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA (three combinations, Fig. 5). No regeneration was observed on MS medium amended with 0.5 and 0.7 mg L⁻¹ BAP, and 0.5 mg L⁻¹ K-NAA (two combinations). The maximum number of 2.33 ± 0.01 bulblets was induced by 0.1 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA. It was followed by the regeneration of 2 0.00 ± 0.03 bulblets using 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA and 1.00 ± 0.01 bulblet induced on 0.3 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA.

Table 3. Average values of callus regeneration percentage, bulblet regeneration percentage, and bulblets number per explant on *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci immature

embryos of different doses of 6-benzylaminopurine (BAP) and potassium salt- α -naphthaleneacetic acid (K-NAA)

BAP (mg L ⁻¹)	K-NAA (mg L ⁻¹)	Callus regeneration (%)	Bulblet regeneration (%)	Number of bulblets per explant
0.1	0.5	0.00 ± 0.00b	6.66 ± 0.02a*	2.33 ± 0.01a
0.3	0.5	0.00 ± 0.00b	6.66 ± 0.01a	1.00 ± 0.05b
0.5	0.5	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00c
0.7	0.5	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00c
0.9	0.5	6.66 ± 0.09†	6.66 ± 0.03 a	2.00 ± 0.03a

*Different alphabetic letters represent statistically significant differences among BAP and K-NAA treatments using LSD test at 0.05 level of significance; ± standard deviation

†6.66 ± 0.9% is taken into consideration in measuring the callus regeneration or viability rate

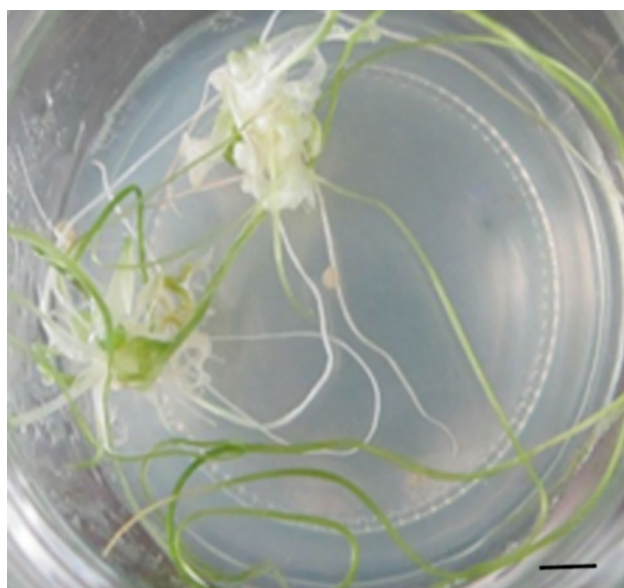


Figure 5. Bulb regeneration on immature embryo explants of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci on Murashige and Skoog medium modified with 0.3 mg L⁻¹ 6-benzylaminopurine (BAP) and 0.5 mg L⁻¹ potassium salt- α -naphthaleneacetic acid (K-NAA). Bar=0.90 cm.

Treatment with MS Medium Modified with 0.1, 0.3, 0.5, 0.7, or 0.9 mg L⁻¹ BAP and 300.0 mg L⁻¹ Putrescine (Five Combinations) MS medium modified with five concentrations of BAP and 300.0 mg L⁻¹ putrescine each (5 combinations) induced 6.66 $\pm$ 0.02–26.66 $\pm$ 1.15% regeneration (Table 4). Callus regeneration was noted on 0.3 mg L⁻¹ BAP and 300.0 mg L⁻¹ putrescine-modified MS medium only, with a regeneration percentage of 13.33 $\pm$ 1.03%. The rest of the treatments did not induce callus. The maximum regeneration percentage was noted on MS medium modified with 0.7 mg L⁻¹ BAP and 300.0 mg L⁻¹ putrescine (Fig. 6). The minimum regeneration percentage was 6.66 $\pm$ 0.02% on MS medium modified with 0.9 mg L⁻¹ BAP and 300.0 mg L⁻¹ putrescine. All bulbs were hyperhydric with long green leaves without roots.

The maximum number of 2.33 $\pm$ 0.02 bulblets was noted at 0.7 mg L⁻¹ BAP and 300.0 mg L⁻¹ putrescine. No bulblets were induced on 0.1 mg L⁻¹ BAP and 300.0 mg L⁻¹ putrescine (Table 4). A low range of 0.66 $\pm$ 0.04–2.33 $\pm$ 0.02 bulblets per explant was obtained.

Regeneration on Cultures Containing MS Medium Modified with 0.1, 0.3, 0.5, 0.7, or 0.9 mg L⁻¹ BAP, 0.5 mg L⁻¹ K-NAA, and 300.0 mg L⁻¹ Putrescine Each The differences in regeneration percentage, callus regeneration percentage, and number of bulblets per explant (Table 5) were statistically significant using five combinations of BAP and K-NAA (0.5 mg L⁻¹) with putrescine (300.0 mg L⁻¹) each. Regeneration percentage values varied between 6.66 and 20.00%. A maximum regeneration percentage of 20.00% was obtained on 0.1, 0.5, or 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine elicitor/booster (three combinations). The minimum regeneration percentage was obtained on 0.7 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine elicitor/booster.

Callus regeneration was observed on MS medium modified with 0.3, 0.7, or 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine (three combinations), whereas 0.1 or 0.5 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine elicitor treatments (two combinations) did not induce callus. These combinations were inhibitory and induced reduced bulblets (0.33 bulblets per explant). However, these treatments were synergetic in terms of inducing an increased number of bulblets per explant. The maximum number (10.33 bulblets per explant) was noted on the 0.3 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine elicitor/booster, followed by 7.66 bulblets per explant on the 0.9 mg L⁻¹ BAP and 0.5 mg L⁻¹ K-NAA with 300.0 mg L⁻¹ putrescine elicitor. All regenerated bulbs had profuse and branched rooting without using any auxin (Fig. 7a, b). The results identified that a combination of BAP and K-NAA with putrescine elicitor had a synergetic effect on bulblet regeneration and rooting. However, the percentage of regeneration never exceeded 20% (in experiments 4 and 5). Non-synchronized ripening of seeds in the umbel could

Table 4. Effects of different concentrations of 6-benzylaminopurine (BAP) and 300.0 mg L⁻¹ putrescine on callus regeneration percentage, bulblet induction percentage, and number of bulblets per explant using immature seeds of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci

BAP (mg L ⁻¹)	Putrescine (mg L ⁻¹)	Callus regeneration (%)	Bulblet regeneration (%)	Number of bulblets per explant
0.1	300.0	0.00 $\pm$ 0.00 b	20.00 $\pm$ 1.01b*	0.00 $\pm$ 0.00e
0.3	300.0	13.33 $\pm$ 1.03 a [†]	13.33 $\pm$ 0.03c	1.66 $\pm$ 0.11b
0.5	300.0	0.00 $\pm$ 0.00 b	20.00 $\pm$ 1.08b	0.66 $\pm$ 0.04d
0.7	300.0	0.00 $\pm$ 0.00 b	26.66 $\pm$ 1.15a	2.33 $\pm$ 0.02 a
0.9	300.0	0.00 $\pm$ 0.00 b	6.66 $\pm$ 0.02c	1.00 $\pm$ 0.01c

*Different alphabetic letters represent statistically significant differences among BAP and putrescine treatments using LSD test at 0.05 level of significance; $\pm$ standard deviation

[†]13.33 $\pm$ 0.35% is taken into consideration in measuring the callus regeneration or viability rate

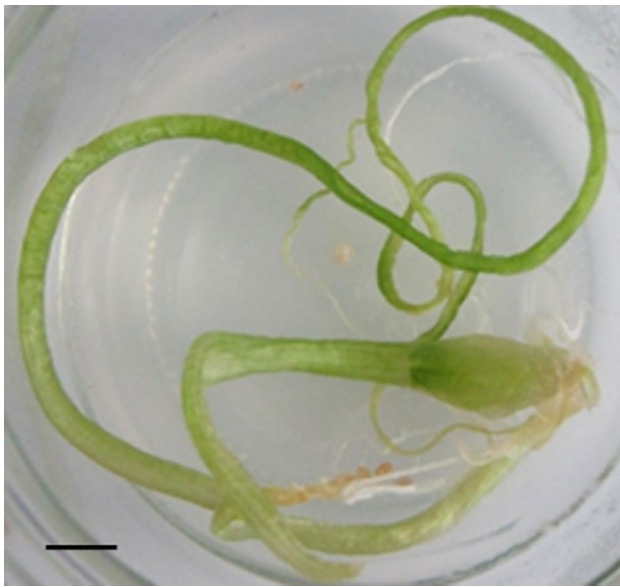


Figure 6. Bulblet regeneration on immature embryo explants of *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci on Murashige and Skoog medium modified with 0.3 mg L⁻¹ 6-benzylaminopurine (BAP) and 300.0 mg L⁻¹ putrescine. Bar=0.90 cm.

be a possible reason that could be related to a lower regeneration percentage on the explants. The endospermic embryos as explants showed their maximum regeneration potential when BAP and K-NAA with putrescine elicitor/booster with a low molecular weight amino acid became active, which is supposed to become active only when K-NAA is used (Okturen and Sonmez 2005; Algul *et al.* 2016). There is no previous study using BAP and K-NAA phytohormones and putrescine elicitor together under *in vitro* conditions on the regeneration of any geophyte previously.

Comparison If we compare the results of hormonal treatments, the maximum number of 12.30 ± 0.69 somatic

embryos or plantlets was noted on 2,4-D containing medium. Similarly, the maximum number of 10.33 ± 0.31 bulblets per explant was noted on BAP and K-NAA with putrescine-containing medium.

Discussion

Regeneration from 2,4-D *A. tuncelianum* does not have synchronization in ripening. The mature seeds are picked three to four times under cultivated conditions. The fixing, staining, and identification of embryos were done following Yilmaz and Khawar (2024). The results are in agreement with Kimani *et al.* (1993) and Goldstein and Shemesh-Mayer (2022). They found irregular and inconsistent regeneration of non-synchronized seeds set in garlic umbels needing three or more picks to collect them. The regenerated bulblets induced from somatic embryos were stout and strong. Many researchers have reported somatic embryogenesis using 2,4-D on many varieties and cultivars of several species using 2,4-D (Meneses *et al.* 2005; Vaziee *et al.* 2024). Non-synchronized ripening of immature embryos in the umbels of *A. tuncelianum* could be a possible cause for the reduced regeneration potential of the explants used in the study.

Regeneration from BAP Singly, or Combinations of BAP and K-NAA, BAP, and Putrescine Along with BAP and K-NAA with a Putrescine Elicitor The regenerated bulbs were hyperhydric. The study consistently demonstrated and underscored the non-synchronized growth of immature embryos in the umbels of *A. tuncelianum* which could be a possible reason for low regeneration. The significant influence of BAP and K-NAA with putrescine elicitor on immature zygotic embryos affected biological performance during developmental and restructuring stages through bulblet organogenesis (Altamura *et al.* 2016). The study consistently demonstrated significant variability and inconsistency in

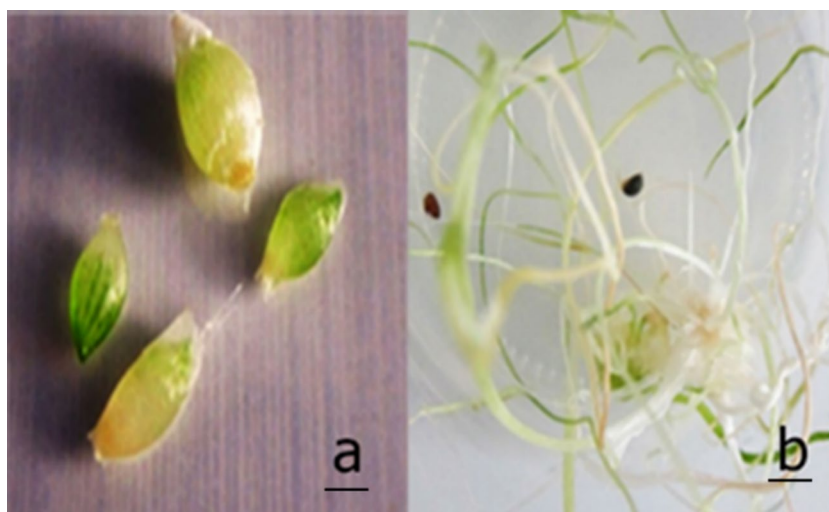
Table 5. Average values of callus regeneration percentage, bulblet regeneration percentage, and number of bulblets per explant on *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci

BAP (mg L ⁻¹)	K-NAA (mg L ⁻¹)	Putrescine (mg L ⁻¹)	Callus regeneration (%)	Bulblet regeneration (%)	Number of bulblets per explant
0.1	0.5	300.0	0.00 ± 0.00c	20.00 ± 1.90a	0.33 ± 0.01d
0.3	0.5	300.0	13.33 ± 0.61 [†] a	13.33 ± 0.19b	10.33 ± 0.31a
0.5	0.5	300.0	0.00 ± 0.00c	20.00 ± 1.23a	0.33 ± 0.01d
0.7	0.5	300.0	6.66 ± 0.02b	6.66 ± 0.01c	1.66 ± 0.11c
0.9	0.5	300.0	6.66 ± 0.05b	20.00 ± 0.89a	7.66 ± 0.16b

*Different alphabetic letters represent statistically significant differences among BAP and K-NAA with putrescine treatments using LSD test at 0.05 level of significance; ± standard deviation

[†]13.33 ± 0.35% is taken into consideration in measuring the callus regeneration or viability rate

Figure 7. Regeneration and rooting of (a) *Allium tuncelianum* (Kollmann) Özhatay, B. Mathew and Şiraneci immature embryos on Murashige and Skoog medium containing 0.9 mg L⁻¹ 6-benzylaminopurine (BAP) and 0.5 mg L⁻¹ potassium salt- α -naphthaleneacetic acid (K-NAA) with 300.0 mg L⁻¹ putrescine (b) showing profuse rooting without using any concentration of any auxin. Bar for panel a = 0.85 cm, Bar for panel b = 0.50 cm.



growth performance among different levels of BAP (Fininkelstein *et al.* 2008; Linkies and Leubner-Metzger 2012). The rest of the immature embryos (the majority) did not induce regeneration. All BAP treatments, with or without putrescine, appeared to have a weak role in inducing bulblet organogenesis on the explants. Although explants induced regeneration, the explants failed to induce satisfactory bulblet regeneration. It was interesting to observe that MS medium modification with putrescine elicitor was effective in the presence of BAP and K-NAA. The putrescine elicitor was not fully supportive with K-NAA. The presence of BAP and putrescine elicitor was rather inhibitory for organogenesis. The accumulation of exogenously supplied putrescine after adding K-NAA to the regeneration medium acted as a booster in the regeneration of the explants (Smith *et al.* 1982). A maximum number of bulblets were regenerated on MS medium modified when BAP, K-NAA, and putrescine elicitor were used together. Metabolomics studies have suggested a positive role and favorable impact of putrescine (Meyer *et al.* 2007) through its involvement in arginine and ornithine decarboxylase activities. This is assumed to support proliferation, growth, and morphogenesis (Masgrau *et al.* 1997; Hanfrey *et al.* 2001). The study explains the role of exogenous application of putrescine in the presence of K-NAA, which remains enigmatic with the need for more studies.

The study used immature seeds (15 d after anthesis) as the explants on water agar and MS medium supplemented with combinations of BAP and K-NAA with and without elicitor putrescine which were insufficient to germinate them in line with Kizil *et al.* (2014) ending up with no germination on water agar (control treatment) that may have negatively influenced germination. However, the process of germination was accelerated after treating the explants with

different plant growth regulators or their combinations to boost regeneration.

Future Direction and Approach There are many gaps in understanding how the maternal environment of monocotyledonous seeds modulated dormancy at every step of growth and development. Currently, used tissue imaging techniques (in life sciences) will also add new knowledge in seed biology in years to come. These techniques will facilitate further understanding of the effects of seed dormancy and seed cell growth. Quantifying these effects could act as powerful tools to eliminate constraints in production technology with practical ends.

The inactivity or low vigor of the embryonic tissues after 2 or 3 wk of anthesis needs more attention through developing new imaging techniques and video microscopy. It will be interesting to note how initiating tissues and different plant growth regulators interact to initiate regeneration and differentiation.

Conclusions

The study analyzes the relationship between 2,4-D treatments and somatic embryogenesis in Tunceli garlic. It also reveals the role of elicitor putrescine, which is highly responsive when used with K-NAA. The observed diversity in the tissue culture response underscores the potential of *A. tuncelianum* which can be used for multiplication of this multiple-purpose plant. Future research should focus on elucidating the genetic and environmental factors limiting the propagation of this endemic and threatened species.

Author contribution SK and KMK conceptualized this idea. SK and UA sources, data curation, and writing original draft preparation; OT helped with resources and data curation; KMK review and regulation. All authors read and accepted the submitted version of the manuscript.

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Data availability Data supporting the finding of this study are available in the article.

Declarations

Conflict of Interest The authors declare no competing interests.

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