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# Effects of Explant Types and Phytohormones on Azarshahr Red Onion Micropropagation

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**ABSTRACT**

The Azarshahr red onion (*Allium cepa* L.), also known as "Germez Azarshahr", is a native variety widely cultivated in northwestern Iran and highly valued for its culinary and pharmacological properties. Seed propagation of this variety is not economical as it results in uneven sowing and excessive consumption of resources such as water. In addition, the yield has significantly decreased due to abiotic and biotic stresses. Since there are no reports on the *in vitro* regeneration of commercially important onion varieties in Iran, this study aimed to evaluate the impact of different explants (mature embryo, basal plate and leaf) and different doses of auxin growth regulators –Naphthaleneacetic Acid (NAA), Picloram, and 2,4-dichlorophenoxyacetic acid (2,4-D)– both individually and together with 6-benzylaminopurine (BAP), on callus induction and regeneration of this variety to improve its use in molecular breeding and genetic engineering programs. The experiments were arranged in a completely randomized design with three replicates, and the data were analyzed using ANOVA and Duncan's multiple-range test ($p \leq 0.05$). The results indicated that the basal plate (50.65%) and mature embryo (40.22%) were the most effective for callus induction in different media. 2,4-D demonstrated superior efficiency in callus induction and production of high-quality embryogenic calli. The maximum callus induction rates were 81.67% for basal plates and 71.33% for mature embryos using Gamborg (B5) medium with BAP + 2,4-D (2+1 mg $L^{-1}$). Furthermore, fifteen shoots were successfully regenerated from each basal plate callus at a frequency of 61.55% using B5 medium with BAP + NAA (1.5+0.1 mg $L^{-1}$). This optimized protocol is suitable for rapid clonal propagation, germplasm preservation, and conducting genetic transformation studies of the Azarshahr red onion variety.

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## 1. Introduction

Onion (*Allium cepa* L.) is a cross-pollinated biennial monocot plant of the Alliaceae family. It is among the most significant vegetable crops in the world and is grown annually for onion production with a global yield of approximately 111 million tons (FAO, 2023). This type of plant is rich in minerals, proteins, vitamins, anthocyanins, and phenolic compounds, which provide health benefits and anti-inflammatory and antimicrobial effects. Due to the existence of these properties, this plant is widely used in herbal medicine and the food industry (Motavallian *et al.*, 2025). The red Azarshahr variety is one of the most important domestic onions, grown mainly in Iran's East Azerbaijan province. It is highly valued for its excellent taste and long shelf life. In recent years, Azarshahr red onion production has declined due to frequent droughts, reduced water levels in Lake Urmia, and salinization of agricultural water in the region (Schmidt *et al.*, 2021). These environmental stresses, along with challenges in conventional propagation methods such as seeds, bulbs, and seedlings, have led to inefficiencies in cultivation. Traditional propagation methods often result in high seed consumption, uneven sowing, and excessive use of resources such as water, fertilizers, and pesticides. Moreover, the biennial growth cycle, the presence of heterozygous genes and difficulties in crossbreeding with other *Allium* species further complicate crop improvement using conventional methods. These limitations hinder the development of

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new varieties that exhibit desirable characteristics such as higher yields, improved quality, and better resistance to environmental stresses (Malik *et al*., 2021). This highlights the need for alternative propagation techniques that are more resource-efficient and environmentally sustainable.

Tissue culture techniques offer a promising solution for the propagation and breeding of *Allium* species, enabling the production of abundant plant material for research and cultivation. These methods have become a cornerstone in plant biotechnology, particularly in the fields of crop improvement, germplasm preservation, and genetic engineering (Hailu *et al*., 2021). While successful *in vitro* regeneration has been achieved in species such as garlic and leek (Mukhopadhyay *et al*., 2005; Yan *et al*., 2009; Nazari *et al*., 2021), research on onion tissue culture, particularly in Iran, remains limited. Factors such as explant type, genotype, and growth regulators play a crucial role in the efficiency of regeneration (Aswath *et al*., 2005). For example, explant type can significantly affect callus induction and shoot regeneration, with basal plates and root apical meristems being particularly responsive in species like garlic and leeks (Watson-Guido *et al*., 2021; Nazari *et al*., 2021). Tissue culture has been widely applied to other *Allium* species, including garlic, leeks, and short-day onions, demonstrating positive results in both regeneration and propagation (Farhadi *et al*., 2017). Table 1 provides a summary of the various plant tissues, varying doses of phytohormones, and media that have been used in the tissue culture of *Allium* species, including onions.

**Table 1. Overview of *in vitro* propagation techniques for *Allium* species**

| Species | Explant | Medium (PGR in mg $L^{-1}$) | Result | Reference |
|---|---|---|---|---|
| *A. chinense* | Basal plate | B5 + (0.1 BA + 1.0 2,4-D)<br>(0.1 BA + 1.0 NAA) | 65.2% callus<br>58.8% Shoot | Yan et al. (2009) |
| *A. hirtifolium* | Basal plate | MS + (1.5 2,4-D + 0.5 BA)<br>MS + (1.0 TDZ + NAA) | 60.06% callus<br>79.30% Shoot | Farhadi et al. (2017) |
| *A. cepa* | Seedling radical | (1.0 2,4-D + 0.5 Kin)<br>(1.5 KIN + 0.125 ABA) | 85.33% callus<br>73.15% Shoot | Manape et al. (2022) |
| *A. cepa* | Shoot apex | (4.0 2,4-D)<br>(1.5 BA + 2.0 glycine ) | 85.3% callus<br>78.1% Shoot | Ramakrishnan et al. (2013) |
| *A. cepa* | Mature embryo | MS + (5.0 Picloram)<br>MS + (1 .0 2,4-D) | 85% callus + 38.9% Shoot<br>78.6% callus + 70.1 % Shoot | Sivanesan et al. (2015) |
| *A. cepa*<br>*A. sativum* | Bulbs | MS + (2 ,4-D + 0.2 KIN)<br>MS + (KIN + NAA) | callus<br>Shoot | Mukhopadhyay et al. (2005) |
| *A. cepa* | Seed | MS + (2-iP + (4-FPA))<br>MS + (2-iP ) | callus<br>Shoot | Tanikawa et al. (1996) |
| *A. ampeloprasum* | Mature embryo + Leaf | MS+ (30 g $L^{-1}$ sucrose + 1.0 2,4-D)<br>MS+ (1 KIN) | callus<br>Shoot | Buiteveld et al. (1993) |
| *A. cepa* | Flower bud | MS + (2 TDZ) | callus | Bohanec et al. (1995) |
| *A. Sativum* | Root tip | MS + (1.1 2,4-D)<br>MS + (1 KIN) | 94.33% Callus<br>95.62% Shoot | Haque (2023) |

B5: Gamborg medium (Gamborg *et al*., 1968). Ms: Murashige Skoog (Murashige and Skoog, 1962).

In these species, callus induction has been effectively achieved using various explants such as seeds, bulbs, shoot apical meristems, ovules, stamens and mature embryos (Mukhopadhyay *et al*., 2005; Ramakrishnan *et al*., 2013). Moreover, the application of phytohormones such as Naphthaleneacetic acid (NAA), Picloram, and 2,4-D in combination with cytokinins such as BAP and kinetin has shown promise in promoting callus formation and regeneration (Sivanesan *et al*., 2015; Haque, 2023). However, there is limited information available on the propagation of Iranian onion cultivars, particularly the red Azarshahr variety, which holds considerable economic and cultural importance. This study aims to evaluate the impact of different explants (mature embryo, basal plate, and leaf) and varying concentrations of phytohormones, including Naphthaleneacetic acid (NAA), Picloram, and 2,4-dichlorophenoxyacetic acid (2,4-D), on callus induction and regeneration in the Azarshahr red onion cultivar. It seeks to identify the optimal combinations of explants and hormones for efficient callus formation and plant regeneration, considering the specific physiological and genetic requirements of this cultivar. By focusing on the Azarshahr red onion, this research addresses an important gap in existing tissue culture protocols, which are typically designed for other *Allium* species and may not be directly applicable to this variety.

Tailoring the approach to the specific characteristics of this cultivar will facilitate the development of an effective tissue culture system. This system is designed to support rapid clonal propagation, germplasm preservation, and genetic transformation, thereby enabling advancements in breeding and genetic studies for this valuable variety.

## 2. Materials and methods

### *2.1. Plant materials*

Seeds and bulbs of Azarshahr red onion were sourced from their indigenous habitats in the Azarshahr region. The experimental procedures were subsequently conducted at the Department of Agricultural Biotechnology, Azerbaijan Shahid Madani University in Tabriz.

### *2.2. Surface sterilization and preparation of explants*

For surface disinfection, onion seed explants and bulbs were first washed under running tap water for 5 minutes. Then, the outer dry scales and roots were removed. The seed explants were then immersed in 70% alcohol for 1 minute, while the bulbs were immersed for 5 minutes. Subsequently, the explants were exposed to a solution containing 2% sodium hypochlorite for 20 minutes. After rinsing four times with autoclaved water, the seeds were immersed in sterilized distilled water and stored at 4°C to facilitate the separation of mature embryos. After 24 hours, the seeds were dissected under sterile conditions with a scalpel blade to isolate mature embryos. After sterilization, onion bulbs were sliced in a laminar airflow hood, cutting basal plates to a thickness of 3–5 mm. Leaf explants were obtained from seedlings grown in hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962).

### *2.3. Basic growth media conditions for callus and shoot formation*

To initiate callus formation and proliferation, B5 medium was supplemented with 1g $L^{-1}$,690 mg $L^{-1}$, 2 mg $L^{-1}$ and 20 g $L^{-1}$ of casein hydrolysate, proline, glycine, and sucrose, respectively (Ramakrishnan *et al.*, 2013). However, for regeneration, only plain B5 medium without additional supplements was utilized. The pH of culture media was adjusted to 5.7±0.1. Before adding 8 g $L^{-1}$ agar, growth regulators, excluding thidiazuron (TDZ), were incorporated into the media. TDZ was added after autoclaving by filter sterilization under a laminar hood. Afterward, for 15 minutes, the media were autoclaved at 121°C to sterilize them.

### *2.4. The impact of various types of explants and phytohormones on callus formation*

The main objective of this study was to evaluate the influence of different phytohormone levels and explant types on three critical parameters: induction of embryogenic callus, fresh weight of callus, and callus size. To achieve this, a completely randomized factorial experiment was conducted, comprising three sets of replicates with ten explants each. The primary factor of the study was varied concentrations of auxins, Picloram, NAA, and 2,4-D, set at three levels (0.5, 1, and 2 mg $L^{-1}$), either independently or in conjunction with BAP at four different levels (0, 0.5, 1, and 2 mg $L^{-1}$).

The second factor involved different explant types, such as leaves, mature embryos, and basal plates ranging from three to four millimeters in size. All treatments were maintained in a phytotron at a temperature of 25±1°C. The obtained calli were sub-cultured to fresh culture medium every three weeks, and after five weeks, the percentage, weight, and size of the calli were measured and documented.

### *2.5. Analysis of morphological characteristics in callus*

Detailed imaging was performed to measure various characteristics such as size, color variations, and overall appearance of callus samples. ImageJ Tool ver. 3 software was used for image analysis. The morphology of the callus using a stereoscopic microscope was investigated, and its type was determined.

### *2.6. The impact of phytohormones on shoot formation*

The objective of the second experiment was to evaluate the ability of yellow friable embryogenic calli of onions to regenerate and induce shoot formation. Various cytokinins, including Thidiazuron (TDZ), Kinetin (KIN), 6-benzylaminopurine (BAP), as well as NAA or Gibberellic acid (GA3), were used for this purpose. Cultures were placed in the phytotron with a light/dark cycle of 16 of hours light (40 µE) and 8 of hours darkness at 25±1°C. Explants were sub-cultured into new culture media every two weeks. After a period

of five weeks, the percentage of regenerated shoots, as well as their length and number, were assessed.

*2.7. Induction of root*

When the regenerated plants in the culture medium reached an appropriate size, they were placed on half-strength MS media including indole3-butyric acid (IBA) (2 mg $L^{-1}$). The cultures were placed in a phytotron with a 16-hour light period followed by an 8-hour darkness interval at 25°C. To acclimate the plantlets to the natural environment, they were removed from the growth medium and their roots were gently rinsed with water. Then, they were placed in plastic pots filled with a blend of agricultural soil, leaf soil, and peat moss mixed in equal parts (1:1:1 ratio).

*2.8. Data analysis method*

Data obtained from the experiments were analyzed using a completely randomized design with three replications. Treatments included three types of explants (mature embryo, basal plate, and leaf) and different concentrations of phytohormones (NAA, Picloram, 2,4-D, BAP, and kinetin). Analysis of variance (ANOVA) was performed to evaluate the effects of explant type, phytohormone concentration, and their interactions on callus induction and regeneration. Mean separation was performed using Duncan's Multiple Range Test at a significance level of $p \leq 0.05$. All statistical analyses were conducted using MSTATC and SPSS software.

## 3. Results and discussion

This study investigated the effects of three auxins (2,4-D, NAA, and Picloram) on callus induction, fresh weight, size, and morphology of Azarshahr red onion. Three types of explants (basal plate, leaf, and mature embryo) were initially tested in combination with these auxins and different concentrations of BAP. The results indicated that leaf explants and the auxin NAA were ineffective for callus induction, as NAA primarily promoted longitudinal growth rather than callus formation. Consequently, basal plate and mature embryo explants were selected for further experiments.

To better understand the specific effects of each auxin and avoid confounding interactions, two separate experiments were designed. The first evaluated the effects of 2,4-D with or without BAP, while the second focused on Picloram with or without BAP, using identical concentration levels in both cases. This approach enabled a detailed analysis of the selected explants' responses to each auxin. By the fifth week, treatments were evaluated based on callus morphology, induction percentage, fresh weight, and size, facilitating the identification of the optimal explant and hormonal combination to produce a callus suitable for regeneration studies.

*3.1. The impact of various types of explants and phytohormones on the formation of callus*

This study investigates the effects of explant type and phytohormonal treatments on callus formation and growth in Azarshahr red onion, highlighting the critical role of both factors in optimizing tissue culture protocols. Analysis of variance revealed that combinations of auxins, with or without BAP, as well as explant type (basal plate and mature embryo) and their interactions, significantly influenced callogenesis (Tables 2 and 3).

**Table 2. Interaction effects of explant types and plant growth regulator combinations (2,4-D + BAP) on callus induction, diameter, and fresh weight in Azarshahr red onion.**

| Source of variation | D.F. | Mean squares | | |
|---|---|---|---|---|
| | | Callus induction | Callus diameter | Fresh weight |
| Hormon | 11 | 1087.686$^{**}$ | 0.083$^{**}$ | 0.301$^{**}$ |
| Explant | 1 | 1960.215$^{**}$ | 0.591$^{**}$ | 0.079$^{*}$ |
| Explant × Hormon | 11 | 520.311$^{**}$ | 0.037$^{**}$ | 0.062$^{**}$ |
| Error | 48 | 35.967 | 0.007 | 0.012 |
| C.V. (%) | | 13.20 | 11.87 | 9.34 |

*Significant in 5% and **Significant in 1%

**Table 3. Interaction effects of explant types and plant growth regulator combinations (Picloram + BAP) on callus induction, diameter, and fresh weight in Azarshahr red onion.**

| Source of variation | D.F. | Mean squares | | |
|---|---|---|---|---|
| | | Callus induction | Callus diameter | Fresh weight |
| Hormon | 11 | 812.005$^{**}$ | 0.395$^{**}$ | 0.009$^{**}$ |
| Explant | 1 | 273.351$^{**}$ | 0.011$^{ns}$ | 0.001$^{**}$ |
| Explant × Hormon | 11 | 106.386$^{**}$ | 0.066$^{**}$ | 0.003$^{**}$ |
| Error | 48 | 15.042 | 0.009 | 0.000 |
| C.V. (%) | | 14.62 | 13.07 | 10.67 |

**Significant in 1% and ns None-significant

Callus induction rates ranged from 12% to 81.6%, underscoring the importance of selecting the appropriate explant and hormonal treatments for successful callus development. Explant selection plays a crucial role in callus induction, as its response is strongly influenced by its genotype and physiological state. Therefore, different types of explants within a species exhibit different levels of responsiveness,

leading to variations in embryogenic callus induction rates (Mostafa *et al*., 2020).

In this study, basal plate explants showed the earliest signs of callus formation, initiating after just two weeks, whereas mature embryos displayed a delayed response, with callus induction beginning after three weeks, demonstrating that they serve as the most efficient and cost-effective source for large-scale onion callus production. Under optimal hormonal conditions (culture medium 20), basal plates achieved the highest induction rate (81.67%), surpassing the 71.33% rate observed in mature embryos. Our findings are consistent with previous studies on Chinese Jiaotou (Yan *et al*., 2009). Similarly, Farhadi et al. (2017) identified basal plate explants as the most effective source for callus formation differentiation in Persian shallot. However, Wu et al. (2015) observed that mature embryos were more suitable for callus differentiation in onion. Differences in meristematic and parenchymal cell numbers may contribute to variations in cell division across different explant types, particularly in the context of callus growth (Neumann *et al*., 2020).

Callus induction from both explants varied significantly depending on the growth regulators used. Although all plant growth regulator treatments resulted in callus induction, 2,4-D at 1.0 mg $L^{-1}$ proved highly effective, particularly when combined with cytokinins (Fig. 1 treatment 20). Picloram at 0.5 mg $L^{-1}$ also demonstrated a high potential for callus induction, especially in mature embryos, achieving an induction rate of 60%. However, higher auxin concentrations (>1 mg $L^{-1}$) negatively affected callus formation, leading to mucilaginous and phenolic calli with reduced induction rates—a phenomenon previously observed in the tissue culture of other *Allium* species, likely due to auxin toxicity or hormonal imbalance (Marinangeli *et al*., 2005; Zheng *et al*., 1999) (Fig. 2). Manape et al. (2022) similarly reported that maintaining accurate auxin-cytokinin ratios is crucial for optimizing embryogenic potential. The findings of this study align with previous reports, as increasing cytokinin levels up to 2 mg $L^{-1}$ in combination with 2,4-D had a positive effect on embryogenic callus formation in both explants. (Dharmayanti *et al*., 2018; Farhadi *et al*., 2017). However, Sivanesan et al. (2015) found that Picloram was more effective than 2,4-D in inducing callus in mature onion embryos.

### *3.2. The impact of phytohormones and explant type on callus fresh weight and size*

The growth and accumulated weight of the callus are crucial parameters for its multiplication, somatic embryo induction, and subsequent plant regeneration. To assess these factors, callus weight and size were measured in the fifth week following the 35-day primary induction phase. Callus fresh weight was significantly influenced by the type of explants, the growth regulators used, as well as their interactions (Tables 2 and 3). Basal plate explants outperformed mature embryos across various hormonal treatments. This may result from their higher endogenous hormone levels and more active meristematic cells, which enhance responsiveness to growth regulators (Mostafa *et al*., 2020). The highest and lowest callus fresh weights in both explants were observed in culture media numbers (15 and 18) and (8 and 9), respectively (Fig. 3). Cytokinins are thought to promote callus formation by reducing cell wall lignification, thereby enhancing callus *in vitro* initiation and development (Ahmad-Dar *et al*., 2021).

In our experiment, Statistical analysis revealed that the mean weight of the callus increases with increasing auxin and cytokinin concentrations up to (1 mg $L^{-1}$), and concentrations above this value resulted in decreased callus weight. The lowest callus weight in both types of explants was associated with hormonal treatments containing Picloram (Fig. 3). In this context, our findings demonstrated that 2,4-D not only stimulated callus initiation but also supported its continued proliferation. These results align with previous studies, such as those by Farhadi et al. (2017) on Persian shallot and Manape et al. (2022) on onion, both of which identified 2,4-D (1.0 mg $L^{-1}$) in combination with BAP (0.5-1 mg $L^{-1}$) as an effective treatment to induce and proliferate embryogenic callus in onion explants. Interestingly, Picloram combined with BAP was the most effective treatment for promoting callus size, which is consistent with the results of Sivanesan et al. (2015), who reported the superiority of Picloram over 2,4-D in stimulating callus diameter growth in *A. cepa* (Fig. 4). Despite its effectiveness in promoting callus size, Picloram treatment was associated with lower fresh weight, suggesting that it stimulates callus formation but does not promote biomass accumulation as effectively as other treatments (Fig. 3).

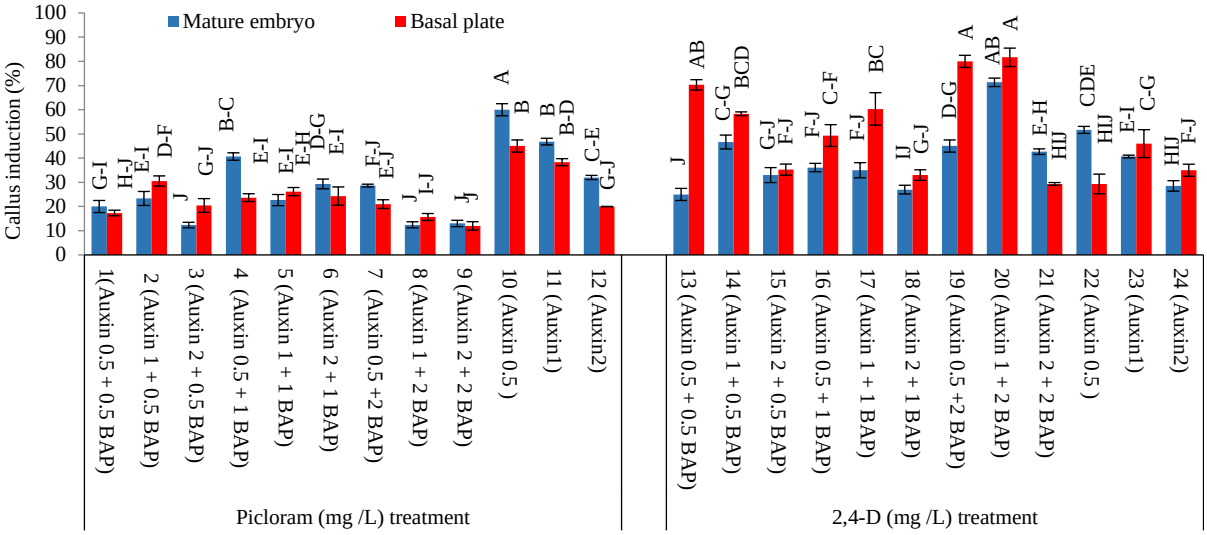


**Figure 1. The impact of phytohormones and explants type on callus induction.**

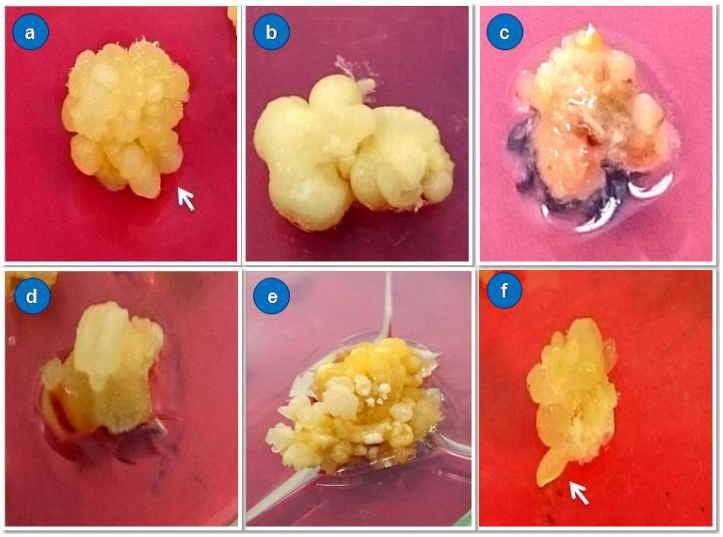


**Figure 2. Different types of *A.cepa* callus. (a) Callus derived from mature embryo (arrow depicts primary embryogenic callus at the globular stage). (b) Non-embryogenic callus derived from mature embryo. (c) Callus with mucilage and phenolics appearance derived from high concentration of auxin. (d) Compact callus derived from basal plate. (e) Callus derived from basal plate (*arrow shows* early heart shaped embryo). (f) Callus derived from basal (*arrow* indicates tadpole- shaped embryo).**

### *3.3. Morphological characteristics of callus*

The Visual characteristics of callus are a crucial indicator of its potential for shoot formation. Morphologically, onion calli exhibited variations in shape, tissue, and color depending on the culture medium used. Various types of embryogenic and non-embryogenic calli, including those containing mucilaginous substances, were observed in different treatments (Fig. 2). Creamy-yellow calli, which exhibited a friable texture, were obtained from explants cultured in media containing Picloram, likely due to the higher hormone concentrations in the induction medium (He *et al*., 1998). In contrast, yellowish calli with a compact texture predominantly developed in media containing 2,4-D (Fig. 2a) which were more suitable for regeneration. Non-embryogenic calli exhibited a soft surface and were larger than embryogenic calli (Fig. 2b,d).

In treatments with high auxin concentrations, calli with mucilaginous and phenolic surfaces were also observed. Two weeks after subculturing, spherical embryos began to form, and by weeks three to four, calli with spherical, heart-shaped, and tadpole-shaped embryos developed (Fig. 2e,f). Studies suggest that hormone requirements are genotype-specific, and optimizing auxin levels in the induction medium requires balancing callus induction efficiency with regeneration frequency. Our observations align with the characteristics of Persian shallot embryogenic callus described by Farhadi et al. (2017) and closely

resemble the developmental patterns reported by Keighobadi et al. (2020) We conclude that 2,4-D in combination with BAP as the optimal growth regulator promotes efficient callus induction and growth, resulting in compact- embryogenic calli capable of plants regeneration.

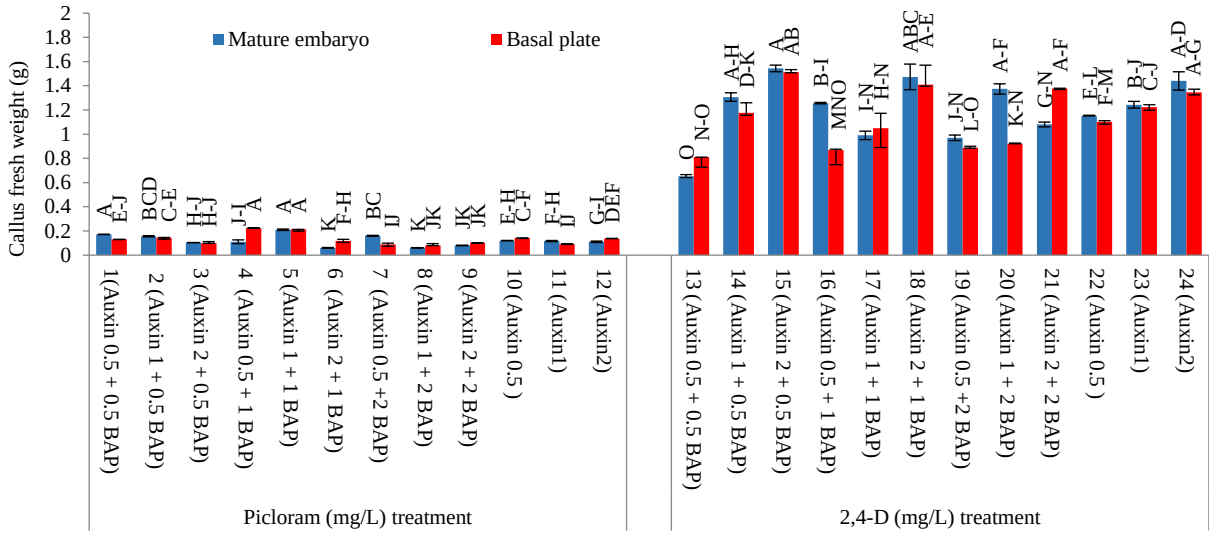


**Figure 3. The impact of phytohormones and explants type on callus fresh weight.**

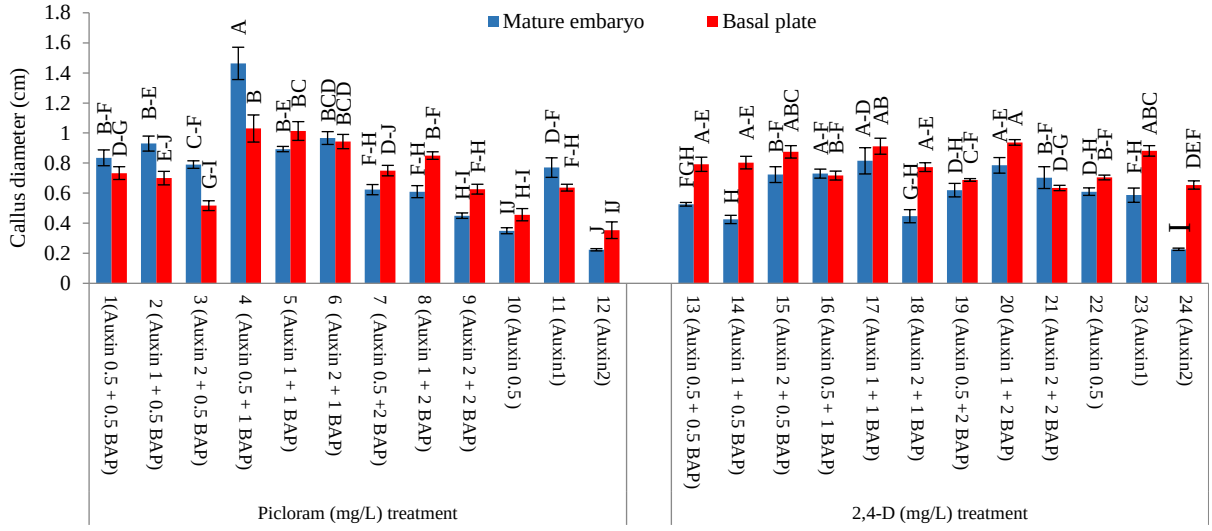


**Figure 4. The impact of phytohormones and explants type on callus size.**

*3.4. The impact of phytohormones on shoot formation*

Cytokinins affect shoot regeneration in *in-vitro* cultures (Tagimanova *et al*., 2024). To further explore their efficacy, we conducted an additional experiment where the best-growing calli were transferred to MS medium containing various concentrations of cytokinins combined with NAA to evaluate their shoot induction potential. Based on previous studies and research on the *Allium* family, we selected hormone types and concentrations commonly reported for successful shoot regeneration. The experimental results demonstrated significant differences in regeneration percentages and shoot numbers among hormonal treatments ($p<0.05$), highlighting the influence of hormone type and concentration on regeneration outcomes. Treatment 1 exhibited the highest regeneration rate (61.55%), followed by Treatments 4 and 5 at 44% and 32%, respectively. These findings align with previous research emphasizing the necessity of optimizing cytokinin and auxin levels for efficient shoot induction (Kristina *et al*., 2023; Malla *et al*., 2015). Notably, shoot length did not vary significantly among treatments, suggesting that shoot initiation and elongation might be regulated by distinct pathways or that elongation is less sensitive to hormonal differences within the tested range (Tables 4 and 5; Fig. 5).

**Table 4. Mean squares for growth regulator effects on shoot regeneration of *A. cepa* callus**

| Source of Variation | D.F. | Shoot induction | Number of shoots per callus | Shoot height |
|---|---|---|---|---|
| Treatments | 2 | 662.76** | 72.33** | 42.11ns |
| Error | 6 | 43.85 | 5.89 | 8.67 |
| C.V. (%) | | 14.44 | 25.1 | 25.72 |

**Significant in 1% and ns None-significant

**Table 5. The impacts of various combinations of phytohormones on shoot formation in the Azarshahr red onion**

| Growth regulators (mg $L^{-1}$) | | | | | | Shoot induction (%) | Number of shoots per callus | Shoot height (cm) |
|---|---|---|---|---|---|---|---|---|
| Medium | NAA | BAP | TDZ | Kin | GA3 | | | |
| 1 | 0.1 | 1.5 | - | - | - | 61.55[a] | 15[a] | 14.66[a] |
| 2 | - | 2 | - | - | - | 0 | 0 | 0 |
| 3 | - | - | - | 2 | - | 0 | 0 | 0 |
| 4 | 0.1 | - | 2 | - | 0.1 | 44[b] | 8.66[b] | 12.33[ab] |
| 5 | 0.1 | 2 | - | 0.5 | 0.1 | 32[b] | 5.33[b] | 7.33[b] |
| 6 | 1 | 2 | - | - | - | 0 | 0 | 0 |
| 7 | 1 | - | 1 | - | - | 0 | 0 | 0 |

Values followed by the same letter within a column indicate they are not significantly different ($p<0.05$) according to Duncan's multiple-range test.

Among the cytokinins evaluated, benzylaminopurine (BAP) consistently demonstrated superior efficacy. It not only promoted earlier signs of regeneration but also resulted in the highest number of shoots (15 per explant) and the longest shoots. This is consistent with prior studies (Tanikawa *et al*., 1996; Passi *et al*., 2018), which identified BAP as more effective than kinetin or other cytokinins, such as TDZ, in onion regeneration. The pronounced response to BAP may be attributed to its stability, higher receptor binding affinity, and its ability to effectively activate shoot-promoting pathways. In contrast, kinetin showed no positive effect on shoot regeneration in our study, reinforcing previous findings on cytokinin variability (Zheng *et al*., 1999; Tubić *et al*., 2016).

The addition of small amounts of auxin (NAA) in combination with cytokinins improved shoot regeneration, supporting the synergistic interaction between these two hormone classes in promoting shoot induction. However, high concentrations of both cytokinins and auxins inhibited regeneration, likely due to hormonal imbalances favoring callus maintenance or root induction over shoot development. This observation was particularly evident in Treatment 6, where elevated auxin levels led to root-like structures instead of shoots, a phenomenon reported in similar studies (Farhadi *et al*., 2017).


**Figure 5. Shoot formation in callus obtained from in vitro cultured basal plates of *A. cepa*. (a) Callus turned to green and began the process of forming shoots. (b) Root-like structures emerged on calluses in treatment 6. (c) Regenerated shoots in treatment 1. (d) Elongation of stems and establishment of root systems. (e) Transferring the plant to a pot.**

The varied callus responses across treatments illustrate the importance of achieving the right hormonal balance for regeneration. In Treatment 2, calli remained green but failed to differentiate, indicating a hormonal environment that favored cell viability but was insufficient for shoot induction. Conversely, in Treatment 6, root-like structures formed, suggesting that elevated auxin levels disrupted the balance required for shoot regeneration. These findings are consistent with previous studies emphasizing the need for precise hormonal optimization to balance exogenous and endogenous

factors for successful shoot induction (A'yun *et al.*, 2021; Plabon *et al.*, 2021). By selecting hormone concentrations and types previously reported for successful regeneration in the *Allium* family, our approach builds on established methodologies while tailoring conditions for the Azarshahr red onion cultivar. This optimization contributes to the development of an efficient tissue culture protocol, supporting broader objectives in clonal propagation, germplasm preservation, and genetic improvement efforts for this valuable species.

## 4. Conclusion

This research revealed that the selection of the best initial explant as well as appropriate phytohormonal treatment is necessary to achieve the highest level of callus induction and regeneration. The best type of explant for callus stimulation was obtained from basal plate explants in a culture medium containing hormonal compounds (1 mg $L^{-1}$ 2,4-D + 2 mg $L^{-1}$ BAP). In contrast, callus formation did not occur in leaf explants and hormonal combinations of NAA and BAP. The highest regeneration efficiency from embryogenic calli was observed in B5 culture medium containing 1.5 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ NAA. The optimized protocol established in this study provides an efficient system for callus induction and regeneration in Azarshahr red onion, which could be applied in various programs such as clonal propagation, germplasm conservation, and molecular breeding through genetic engineering.

## Abbreviation

NAA: Naphthaleneacetic acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid; BAP: 6-Benzylaminopurine; B5: Gamborg's medium; TDZ: Thidiazuron; MS: Murashige and Skoog medium; KIN: Kinetin; GA3: Gibberellic acid; IBA: Indole3-butyric acid; 2 iP: N6 (2-isopentenyl); 4-FPA: 4-Fluorophenylacetic acid.

## Conflict of interests

All authors declare no conflict of interest.

## Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

## Consent for publications

The manuscript has been read and approved for submission here by all the named authors.

## Availability of data and material

All the data are embedded in the manuscript.

## Authors' contributions

A.S.A.M. contributed to writing the original draft, review and editing, conceptualization of the study, methodology development, data curation, and investigation. M.P. provided supervision, conducted writing review and editing, administered the project, contributed to conceptualization, and participated in investigation and methodology development. A.T. validated the findings of the study.

## Informed consent

The authors declare not to use any patients in this research.

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