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Optimization of MS liquid medium to increase saffron cell biomass

Somaye Amini[1,2], Seyed Mahdi Ziaratnia[3,*] and Nasim Adibpour[3,4]

1, Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
2, Badiee Saffron Production Agricultural Company, Mashhad, Iran
3, Department of Food Biotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran
4, Grownida Innovation Center, Mashhad, Iran

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***Corresponding author:**
Department of Food Biotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

Email: m.ziaratnia@rifst.ac.ir

ABSTRACT

Purpose: The objective of this study was to enhance the MS-based medium to maximize cell biomass obtained from saffron corm within a liquid culture system. **Research Method:** The initial experiment employed a factorial design to examine three key factors influencing cell biomass: total nitrogen concentrations (20, 40, and 60 mM), ammonium/nitrate ratios (100:0, 75:25, 50:50, 25:75, and 0:100), and sucrose concentrations (3, 6 and 9%). In the second experiment, we evaluated the effect of a basal liquid MS medium enriched with L-glutamine, L-cysteine, PVP, chitosan, and KH_2PO_4 on cell biomass. The optimized medium from Experiment 1 was further enhanced based on the results obtained from Experiment 2 in order to increase cell biomass. Its effects were then compared to those of the standard MS medium by measuring fresh weight of cells over a six-week period in Experiment 3. **Findings:** Results from the initial experiment demonstrated a significant increase in cell biomass (3.37 g) when using 3% sucrose with nitrate instead of ammonium. Lowering the nitrogen concentration from 60 mM to 40 mM significantly improved cell growth. Additionally, both PVP and KH_2PO_4 contributed to increased saffron cell fresh weight in the second experiment. However, it was noted that even at low concentrations, chitosan application significantly enhanced cell death. The findings from the third experiment revealed that the modified MS culture medium, combined with potassium phosphate, significantly enhanced cell biomass growth compared to the standard MS medium. **Research Limitations:** No limitations were identified during this study. **Originality/Value:** This study evaluated key factors affecting cell biomass in saffron, but future research should explore the production of saffron metabolites under these influences.

INTRODUCTION

Saffron, derived from the dried stigmas of the *Crocus sativus* L. flower, is recognized as the world's most valuable spice and medicinal plant. Due to its low average yield, extremely short flowering period, and limited cultivation in only a few countries, the potential for enhancing saffron production appears constrained (Lachguer, 2025; Sharma & Piqueras, 2010). Additionally, the low level of mechanization in various stages of saffron production such as harvesting, separating flower components, and drying combined with high labor costs, significantly contributes to its status as the most expensive spice globally. Therefore, increasing saffron production is a viable strategy to reduce its price. This objective can be achieved through several approaches, including improving crop yields, implementing mechanization in harvesting and processing, and utilizing biotechnological techniques like tissue and cell cultures (Gresta et al., 2009; Husaini et al., 2010; Golmohammadi, 2014).

Cell suspension culture has emerged as a promising alternative to conventional intact plant culture, establishing itself as a commercially viable system for the production of valuable compounds (Bapat, 2023; Gopi & Vatsala, 2006). Success in this method relies on the optimization of the growth medium to maximize cell biomass (Xu et al., 2011). A critical aspect of this optimization involves the sources of carbon and nitrogen (George et al., 2008). Nitrogen is essential for plant metabolism, serving as a foundational component in the synthesis of proteins, nucleic acids, chlorophyll, coenzymes, phytohormones, and secondary metabolites (Hawkesford et al., 2012). This element is typically supplied as ammonium (NH_4^+) and nitrate (NO_3^-) in plant cell culture media (Zhu et al., 2014). Both the overall nitrogen content and the specific proportions of ammonium and nitrate in the medium formulation are important factors influencing growth (Shi et al., 2023). The total nitrogen concentration in basal media ranges from 20 mM to 60 mM, depending on the formulation (Zhu et al., 2014).

Carbohydrates, alongside nitrogen, play a vital role in in vitro cultures by serving both as an essential energy source and an osmotic agent. Research consistently demonstrates that sucrose is the most effective carbohydrate for plant cell cultures due to its compatibility with various plant cells and its affordability and widespread availability (George et al., 2008).

In certain instances, researchers incorporate elicitors, such as chitosan, into plant culture media to enhance cell biomass growth or stimulate the production of secondary metabolites (Amirkavei Najafabadi, 2020; Shah et al., 2024). Chitosan, a versatile biopolymer composed of D-glucosamine units, is found in the cell walls of fungi and the exoskeletons of arthropods. Plants treated with chitosan exhibit defensive responses, including increased antioxidant enzyme activity, phenolic compound accumulation, and flavonoid release (Shah et al., 2024). Moreover, chitosan concentration significantly influences cell growth in suspension culture (Mofid Bojnoordi, 2022).

Optimizing the culture medium by precisely adjusting organic and mineral components is often employed to enhance cell biomass production significantly. Potassium phosphate (KH_2PO_4) is one chemical compound whose concentration is critical for promoting cell growth. This compound is essential for plant reproduction, as it contains two key elements phosphorus and potassium that are vital for optimal growth and development. Phosphorus plays a pivotal role in various physiological processes, including cell division and callus development, while potassium contributes significantly to overall plant vitality (Chewapanich et al., 2021). Numerous studies have explored the effects of various compounds, including different amino acids (Amirkavei Najafabadi et al., 2020), polyvinylpyrrolidone (PVP) (Ranandi et al., 2022), and mineral substances like potassium (Liu & Zhong, 1996), on cell growth and secondary metabolite production in plant cell cultures. Additionally, Amini et al.

investigated how optimizing aeration and pH within a bioreactor can enhance both cell biomass growth and crocin metabolite production in saffron cell cultures (Amini et al., 2022). Unpublished research from the same group has assessed the influence of various carbohydrates, diverse culture media, and both biological and non-biological elicitors on crocin metabolite production and cell biomass in saffron suspension cultures.

This study aims to maximize cell biomass yield by evaluating the effects of total nitrogen at three levels, derived from NH_4^+ and NO_3^- in various ratios, within MS medium enriched with different sucrose concentrations on the growth of *C. sativus* cells in suspension culture. Subsequently, the effects of various amino acids (L-glutamine and L-cysteine), as well as PVP, chitosan, and KH_2PO_4, will be investigated. Ultimately, the promising results from these initial experiments will be integrated into a comprehensive analysis to assess their collective influence on the saffron cell growth index.

MATERIALS AND METHODS

Saffron corms were harvested in May from a research farm at the Research Institute of Food Science and Technology, Mashhad, Iran to prepare explants for callus induction (Ziaratnia & Amini, 2021). Callus induction was happened using Murashige and Skoog (MS) solid medium enriched with 2,4-D (2 mg/L), BAP (4 mg/L), and sucrose at 3%. The cultures were cultivated under the controlled conditions at a temperature of 25 ± 2˚C in dark. For the enhancement of friable biomass, proliferated calli were subculture in MS medium with similar compositions, except for the hormonal combination, which was reduced to 0.5 mg/L for both 2,4-D and Kinetin (Amini et al., 2023). To achieve a homogeneous population of cells, friable calli were transferred to MS liquid medium and subculture at four-week intervals over three months. To create a cell suspension culture, 0.5 g of cells was inoculated into a 100 ml flask containing 20 ml of medium.

First experiment (optimization of nitrogen and carbohydrate levels)

To evaluate the effects of carbon and nitrogen sources, as well as their concentrations, on cell biomass, the total nitrogen content of MS liquid medium and the ratio of NH_4^+ to NO_3^- were adjusted at three levels (20, 40, and 60 mM) across five ratios of NH_4^+ to NO_3^- (100:0, 75:25, 50:50, 25:75, and 0:100 percent). Subsequently, sucrose was added at three concentrations (30, 60, and 90 g/L). All treatments were supplemented with constant hormonal combinations of 2,4-D (0.5 mg/L) and kinetin (0.3 mg/L) and were conducted in three replicates. For each replicate, 0.5 g of cells was inoculated into 20 mL of culture medium contained in a 100 mL Erlenmeyer flask. The flasks were then placed on a rotary shaker at 120 rpm and maintained at a temperature of 23-25 °C. After four weeks, the interaction effects among factors were evaluated based on cell fresh weight. Statistical analysis was performed using a factorial experiment in a completely randomized design (CRD).

Second experiment (optimization of amino acids, PVP, chitosan, and KH_2PO_4 levels)

The objective of this experiment was to assess the effects of various substances, including L-glutamine, L-cysteine, PVP, chitosan, and KH_2PO_4, on the optimization of cell biomass production. The experiment was conducted using a basal MS medium, which was supplemented with a consistent hormonal mix while varying the concentrations of the additives: PVP (10, 100, 500, 1000, and 1500 mg/L), L-glutamine (50, 100, 150, 200, and 250 mg/L), L-cysteine (50, 100, and 150 mg/L), chitosan (10, 25, 50, 100, and 150 mg/L), and KH_2PO_4 (2.5 and 5.0 mM). Cell culture method and storage conditions were similar to the previous experiment (continuous darkness, rotary speed 120 rpm and temperature 23-25°C).

After a period of four weeks, data on the fresh weight of separated cells were collected and subjected to statistical analysis. In this part, the treatments were organized according to a Completely Randomized Design (CRD).

Third experiment (Comparison of modified MS medium from first and second experiment with basal MS medium)

To enhance the composition of the culture medium for saffron cell growth, the modified MS medium from the initial experiment was enriched with the most promising compounds identified in the second experiment, including polyvinylpyrrolidone and potassium phosphate. This refined medium was then compared to the standard MS medium, which served as the control treatment. In this phase of the experiment, we investigated the effects of both modified and standard MS media, each supplemented with different concentrations of polyvinylpyrrolidone (PVP) (0, 500, and 1500 mg/L) and potassium phosphate at levels of either 0 or 5 mM. After four weeks, we measured the fresh weight of the separated cells across three replicates and conducted a statistical analysis using a factorial experiment under a Completely Randomized Design (CRD) to evaluate the results.

Statistical analysis

In the first and third experiments, the interaction effects among factors were evaluated based on cell fresh weight and statistically analyzed using a factorial experiment under a Completely Randomized Design (CRD). Mean comparisons were subsequently conducted using the Duncan method. The treatments in the second experiment were also organized according to a Completely Randomized Design (CRD). All experiments were conducted with three replicates.

RESULTS AND DISCUSSION

Optimization of nitrogen and carbohydrate levels

The results of analysis of variance showed that the concentration of sucrose, total nitrogen levels, and the NH_4^+/NO_3^- ratio significantly influenced the fresh weight of corm-derived cells in suspension culture of saffron ($p \leq 0.05$) (Table 1).

Table 1. Analysis of variance of the effect of different levels of sucrose, total nitrogen and ammonium/nitrate ratio on cell fresh weight in saffron suspension culture.

Source	DF	Mean square	p-value
Sucrose levels	2	26.79	0.000
Total nitrogen	2	0.64	0.000
Amonium/Nitrate ratio	4	15.41	0.000
Replication	3	0.07	0.163
Sucrose levels*Total nitrogen	4	0.26	0.000
Sucrose levels*Amonium/Nitrate ratio	8	3.21	0.000
Total nitrogen*Amonium/Nitrate ratio	8	0.29	0.000
Sucrose levels*Total nitrogen*Amonium/Nitrate ratio	16	0.16	0.000
Error	132	0.04	
Total	179		

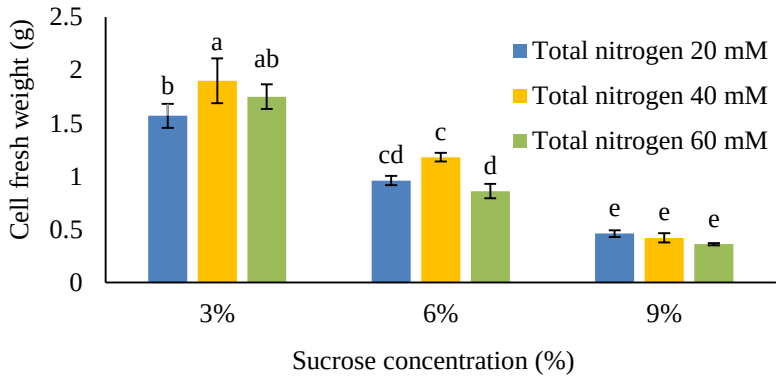

Fig. 1. The interaction effect of sucrose concentration and total nitrogen on fresh weight of cells in saffron cell suspension culture. (Means with the same letter do not demonstrate a significant difference, $p>0.05$).

According to the findings illustrated in Figure 1, cell biomass decreases with increasing sucrose concentration in the culture medium. The liquid MS medium enriched with 3% sucrose and 40 mM nitrogen yielded the highest enhancement in cell biomass, reaching an impressive 1.9 g. However, the culture medium with 60 mM nitrogen and 3% sucrose exhibited no significant difference in cell growth (1.74 g) compared to the previously mentioned treatment. Interestingly, elevating the sucrose concentration to 9% resulted in a complete absence of cell growth across all total nitrogen concentrations.

Figure 2 illustrates the interaction effect between sucrose concentration and the ammonium/nitrate ratio on the fresh weight of saffron cells. As the results show, when nitrogen in the culture medium is supplied exclusively from the nitrate source in combination with 3% sucrose, it yields the highest cell growth at 3.37 g. Conversely, in a culture medium with a similar sucrose concentration (3%) where the nitrogen source was changed from nitrate to ammonium, cell growth substantially decreased to 0.48 g. A declining trend in cell growth was observed at sucrose concentrations of 6% and 9% with the replacement of nitrate by ammonium, similar to the 3% sucrose concentration.

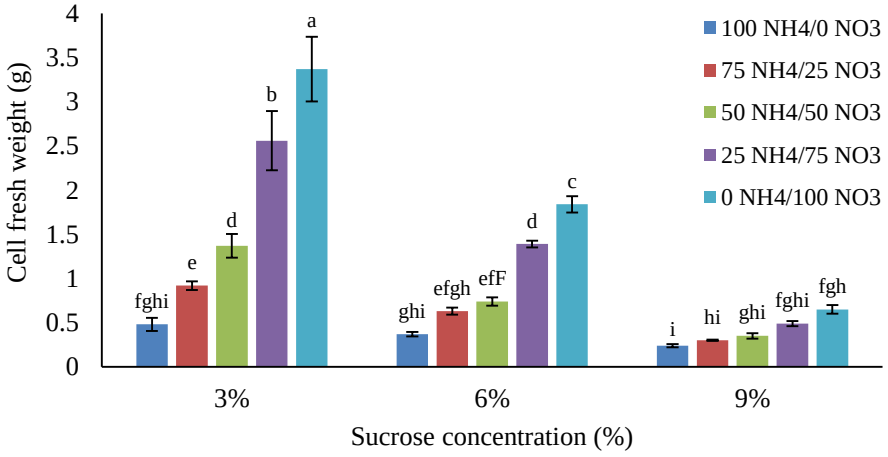

Fig. 2. The interaction effect of sucrose concentration and ammonium/nitrate ratio on fresh weight of cells in saffron cell culture. (Means with the same letter do not demonstrate a significant difference, $p>0.05$).

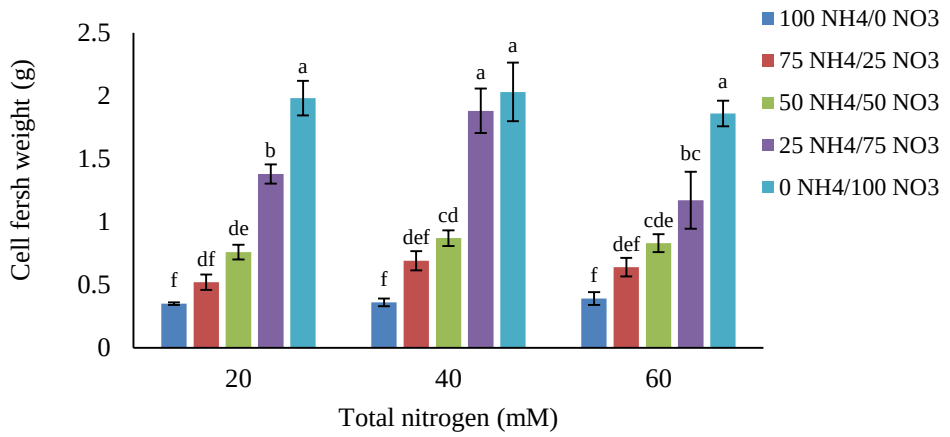

Fig. 3. The interaction effect of total nitrogen and ammonium/nitrate ratio on fresh weight of cells in saffron cell culture. (Means with the same letter do not demonstrate a significant difference, $p>0.05$).

The results presented in Figure 3 indicate that in the free ammonium culture medium, where only nitrate was used as the nitrogen source, the highest cell growth was achieved. Notably, cell biomass production was not affected by the total nitrogen concentration. When ammonium was entirely removed from the culture medium and replaced with 100% nitrate at all three total nitrogen concentrations (20, 40, and 60 mM), the highest cell growth was observed. As shown in Figure 3, if the total nitrogen concentration in the medium is 40 mM, 25% of this nitrogen can be sourced from ammonium without diminishing cell growth.

The findings of this study reveal that reduced sucrose concentrations in the culture medium significantly promote the growth of saffron cell biomass. This improvement can be attributed to osmotic pressure in the medium, where lower levels of carbohydrates facilitate greater water accessibility and nutrient absorption from the substrate (Suan See et al., 2011; Ghosh et al., 2016). Numerous studies have indicated that elevated sucrose levels can lead to increased osmotic pressure, subsequently hindering cell growth. An interesting observation was made with *Hypericum perforatum* cells, where supplementation with 3% sucrose resulted in optimal biomass; however, concentrations exceeding 9% notably suppressed growth (Cui et al., 2010). The strategic application of moderate sucrose concentrations to enhance cell biomass has been consistently supported in various research efforts. For example, cell suspension cultures of *Withania somnifera* exhibited the highest biomass in a medium containing 5% sucrose (Sivanandhan et al., 2013). Additionally, a study focusing on *Prunella vulgaris* revealed that maximum biomass and secondary metabolite production occurred with sucrose concentrations ranging from 2% to 2.5% (Fazal et al., 2016).

The study's findings also indicate that saffron cells preferentially utilize nitrate (NO_3^-) over ammonium (NH_4^+) for optimal cell multiplication and growth. Researchers have identified nitrate and ammonium as primary sources of nitrogen for plants (Hachiya et al., 2012). While it is widely recognized that a combination of NO_3^- and NH_4^+ nutrition significantly enhances plant growth and nutrient accumulation (Lu et al., 2009; Chang et al., 2010), studies indicate that nitrate ions are more readily absorbed by plants in the soil (Maximo et al., 2015). The preference for NO_3^- over NH_4^+ has been documented in several plants for the purpose of cell proliferation (Hachiya et al., 2012; Matsubayashi & Sakagami, 1998). For example, the most significant proliferation of *Asparagus* cells was observed in a medium with an ammonium-to-nitrate ratio of 0:30 mM (Matsubayashi & Sakagami, 1998). When ammonium serves as the sole nitrogen source, plant growth is notably inhibited compared to when nitrate is the only nitrogen source this phenomenon is widely recognized as

NH_4^+ toxicity (Hachiya et al., 2012). The toxicity of NH_4^+ can be attributed to various factors, including nutrient deficiencies caused by impaired cation uptake, disruptions in osmotic balance, alterations in phytohormone levels, disturbances in nitrogen enzyme metabolism, and fluctuations in several key metabolites. Research has shown that utilizing a combination of nitrate and ammonium as the primary nitrogen source significantly reduces ammonium toxicity in various plant species, including those that produce amino acids and organic acids (Garnica et al., 2009; Britto & Kronzucker, 2002). However, in the present study, the complete removal of ammonium and its substitution with nitrate resulted in the most significant improvement in cell growth.

Total nitrogen is a crucial factor influencing cell biomass in plant suspension cultures (Heidari et al., 2020). This study demonstrates that a moderate total nitrogen concentration of 40 mM significantly enhances saffron biomass production. Previous research supports this finding, with Veliky and Rose (1973) noting that the optimal nitrogen level for carrot cell suspension cultures is approximately 500 mg per liter, equivalent to around 36 mM. Additionally, another study found that a total nitrogen level of 30 mM was effective for individual cell proliferation (Veliky & Rose, 1973).

Optimization of amino acids, PVP, chitosan, and KH_2PO_4 levels

The second part of this study investigated the impact of various supplements on the growth of saffron cell mass within a basal MS culture medium. The analysis of variance revealed that various treatments, including different compounds and concentrations, significantly affected the growth of saffron cell biomass in suspension culture (Table 2).

Significant findings regarding the effects of these chemical factors and their concentrations on the fresh weight of saffron cell biomass are presented in Figure 4. While the addition of amino acids, such as L-glutamine, along with chitosan, yielded no noticeable benefits, but the PVP into the medium had a significantly positive effect on saffron cell growth. The data presented in this figure indicates that MS culture media enriched 500 and 1500 mg/L of PVP markedly enhanced cell biomass growth in comparison to the standard basal MS culture media. Conversely, MS media supplemented with 150 mg/L of chitosan markedly reduced cell biomass growth when compared to the standard MS medium. The other treatments analyzed in this section of the study showed no significant difference in biomass growth compared to the standard MS culture medium.

Table 2. Analysis of variance of the effect of different compound and concentration on cell biomass in saffron suspension culture.

Source	DF	Mean square	p-value
Replication	3	0.05	0.402
Treatment	21	0.58	0.000
Error	63	0.05	
Total	87		

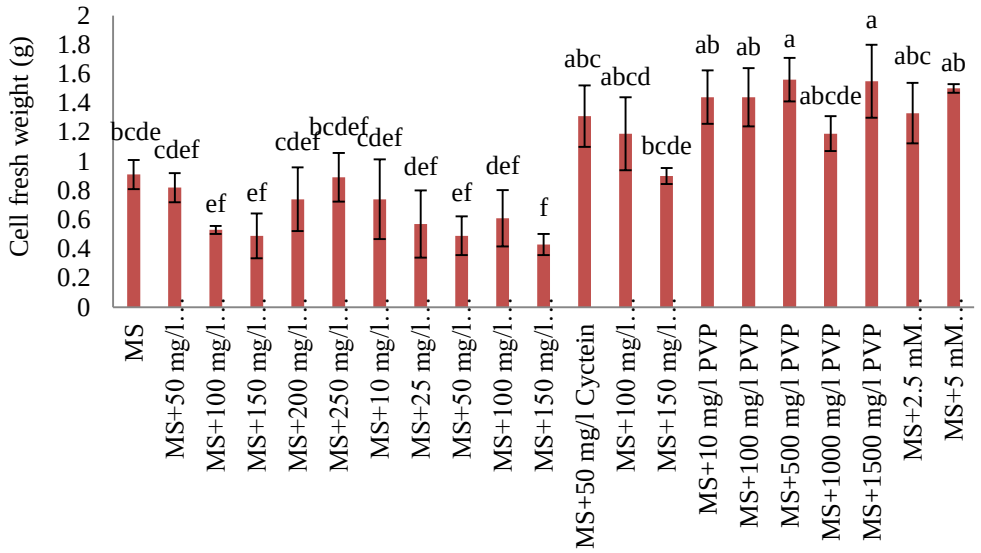

Fig. 4. Comparison of basal MS medium vs. MS enriched with glutamine, chitosan, cysteine, PVP, and potassium phosphate on saffron cell growth in suspension culture. (Means with the same letter do not demonstrate a significant difference, $p>0.05$).

Fig. 5. Evaluation of cell status for various test treatments conducted after a period of 6 weeks. (A) MS with 5 mM potassium phosphate; (B) MS with 500 mg/L PVP; (C) MS with 1500 mg/L PVP; (D) MS with 150 mg/L chitosan.

Figure 5 presents a comparison of saffron cells under various treatments, highlighting their distinct appearances. Notably, the image reveals that chitosan caused burn damage to the cells (Fig. 5D), while cells cultivated in the presence of PVP (Fig. 5B & 5C) or potassium phosphate (Fig. 5A) exhibited healthy growth without any signs of burn damage.

Comparison of modified MS medium from first and second experiment with basal MS medium

The third segment of this research focused on enhancing saffron cell biomass in suspension culture using a modified MS culture medium. This optimized medium, derived from the results of the first experiment, consisted of the basal MS formulation supplemented with 3% sucrose, 40 mM total nitrogen, and an ammonium/nitrate ratio of 0:100. Additionally, it included specific components identified in the second experiment: varying concentrations of PVP (0, 500, and 1500 mg/L) and potassium phosphate (0 and 5 mM). The objective was to determine the most effective composition for increasing cell biomass, building on the findings from the primary and secondary experiments.

The results of analysis of variance showed that the type of basal culture medium (basal MS or modified MS) significantly affected cell biomass growth. Also, the interaction effect of the type of culture medium and potassium phosphate as well as the interaction effect of PVP and potassium phosphate on biomass growth are significant at the 95% probability level (Table 3).

Figure 6 illustrates the comparison between two types of culture media: the basal MS medium and the modified MS medium, both examined for their influence on saffron cell growth in the presence of 5 mM potassium phosphate or its absence. The basal MS medium features a total nitrogen concentration of 60 mM, derived from ammonium and nitrate in a ratio of 33.3:66.6. In contrast, the modified medium reduces the total nitrogen to 40 mM, utilizing only nitrate as the nitrogen source and completely omitting ammonium. Both media maintain a sucrose concentration of 3%. As shown in Figure 6, the modified medium significantly enhances cellular growth compared to the basal medium, particularly when supplemented with 5 mM potassium phosphate. Interestingly, the addition of potassium phosphate to the basal MS medium fails to promote cell growth and appears to exert an inhibitory effect, although this inhibition is not statistically significant.

Table 3. Analysis of variance of the effect of basal medium, PVP and potassium phosphate on cell fresh weight in saffron suspension culture.

Source	DF	Mean square	p-value
Basal medium	1	2.22	0.000
PVP	2	0.12	0.110
Potassium phosphate	1	0.03	0.395
Replication	3	0.07	0.259
Basal medium*PVP	2	0.14	0.075
Basal medium*Potassium phosphate	1	0.62	0.002
PVP*Potassium phosphate	2	0.43	0.001
Error	35	0.05	
Total	47		

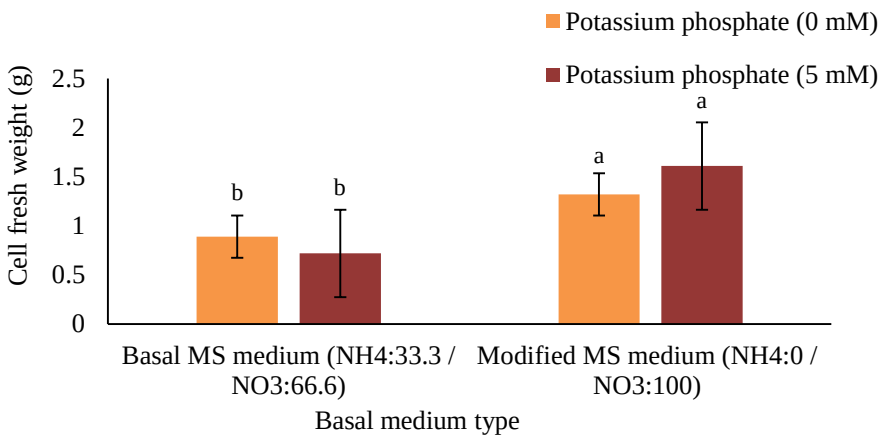

Fig. 6. The interaction effect of basal and modified MS medium and potassium phosphate concentration on fresh weight of cells in saffron suspension culture. (Means with the same letter do not demonstrate a significant difference, *p>0.05*).

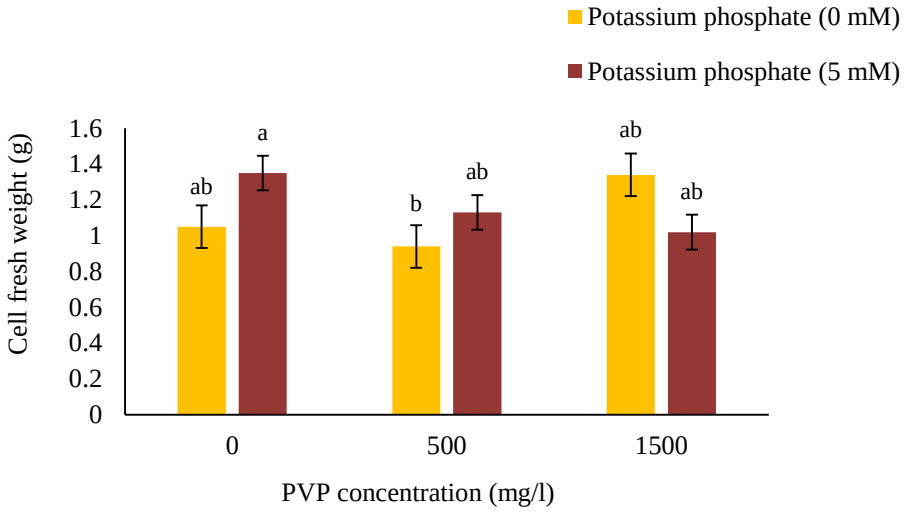

Fig. 7. The interaction effect of PVP concentration and potassium phosphate on fresh weight of cells in saffron cell culture. (Means that share the same letter do not demonstrate a significant difference, *p>5%*).

Figure 7 demonstrates the interaction effect of PVP and potassium phosphate on the cellular growth of saffron in suspension culture. The findings reveal that the most significant cell growth was achieved in the media devoid of PVP but enriched with 5 mM potassium phosphate. Notably, there were no substantial differences observed when comparing this treatment to others.

The second and third sections of this study explored the impact of various compounds on saffron cell biomass growth. Among these, PVP emerged as a significant contributor to cell biomass enhancement. Researchers have indicated that PVP inhibits oxidation by absorbing phenolic compounds released from damaged cells, thereby preventing browning (Cai et al., 2020). The results of this study further validated the previous findings, demonstrating that cell grown in a culture medium enriched with PVP retained their original clarity and displayed enhanced cell biomass. One of the compounds that caused burn symptoms in cells at all concentrations, even at low concentrations, was the compound chitosan. Research findings indicate that incorporating chitosan as an elicitor into culture medium can enhance the

synthesis of secondary metabolites; however, this addition may also reduce cell viability and hinder growth due to the induction of lipid peroxidation, particularly at high concentrations (Mofid Bojnoordi, 2022). While this study primarily focused on increasing cellular biomass without assessing secondary metabolites, the significant darkening of the cells suggests potential synthesis of phenolic compounds resulting from chitosan's influence. Moreover, the significant reduction in cell growth within chitosan-rich cultures strongly indicates cellular damage. Another group of researchers demonstrated that, although chitosan increases secondary metabolite synthesis in plant cell cultures, it also reduces cell biomass (Farjaminezhad & Garoosi, 2021), consistent with the results obtained in this study. In 2010, Keng et al. reported contradictory findings regarding the use of chitosan in *Eurycoma longifolia* cell culture, proposing a concentration of 100 mg/L of chitosan as a growth stimulant for promoting cell biomass.

In this study, potassium phosphate emerged as a significant contributor to the growth of saffron cell biomass. A prior study conducted by Supatmi and Endang (2007) examined the concentration of potassium phosphate in MS medium and found that lower levels of KH_2PO_4 markedly inhibited the growth of callus tissue in Pule Pandak plants. This research underscores the essential role of optimal potassium phosphate concentration in enhancing cellular growth (Supatmi & Endang, 2007).

Researchers identified a noteworthy correlation between the consumption of nitrogen as nitrate in the culture medium and the levels of potassium present. Their findings revealed that an increase in potassium content in the culture medium correlated with a rise in nitrogen uptake by the cells, subsequently leading to enhanced biomass growth and greater synthesis of metabolites (Liu & Zhong, 1996). This correlation likely explains the enhanced cell growth observed in this study in the presence of potassium phosphate.

CONCLUSION

In conclusion, the findings of this study demonstrate that modifying the concentrations of carbohydrates and nitrogen sources in the culture medium significantly impacts saffron cell growth. Notably, employing nitrate as the exclusive nitrogen source resulted in enhanced saffron cell biomass. The research identified the optimal medium for maximizing saffron cell biomass as a modified MS medium, with nitrate serving as the sole nitrogen source. Further growth improvements can be achieved by incorporating compounds such as monopotassium phosphate at 5 mg/L. Additionally, reducing the total nitrogen concentration in the MS culture medium from 60 mM to 40 mM and adjusting the sucrose concentration to 3% will further promote the growth of saffron cells in suspension culture. This optimized protocol provides a valuable approach for scaling up the cultivation of *Crocus sativus* L. cells, paving the way for future studies aimed at enhancing saffron secondary metabolites within a cell suspension system.

Conflict of interest

Authors hereby declare that there is no conflict of interest.

Author contributions

In this study, Ziaratnia conceptualized the experiments, while Amini and Adibpour executed the experiments, conducted data analysis, and authored the manuscript. All authors reviewed and approved the final version of the paper.

Data availability statement

The datasets generated during and/or analyzed during the current study are available through the corresponding author based on reasonable request.

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