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In Vitro Optimization of Callus Induction, Somatic Embryogenesis, and Secondary Metabolite Production in *Iris pseudacorus* L.

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ABSTRACT

Yellow flag (*Iris pseudacorus* L.) is an important endangered plant, commonly cultivated for its aesthetic and medicinal value. Optimization of in vitro culture conditions, callus development, somatic embryogenesis, and plant regeneration constitutes a critical initial step for successful propagation. This study investigated the effects of various concentrations (low to high) of 2,4-D and kinetin on callus formation parameters and secondary metabolite production. Conducted as a factorial experiment with a completely randomized design, it included 15 treatments (plant growth regulators) under different light conditions (light and darkness). Results indicated that darkness had a more pronounced positive effect on callus development than light. The highest total phenol content (47.95 mg 100 g^{-1}) was found in the treatment of 7.5 mg L–1 2,4-D + 0 kinetin. Additionally, the treatment of 2.5 mg L^{-1} 2,4-D + 0.5 mg L–1 kinetin yielded the highest flavonoid content (72.08 mg g^{-1}), while 10 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin produced the lowest. Light conditions favored greater flavonoid production compared to darkness. Treatment interactions did not significantly affect anthocyanin production. The highest level of anthocyanin (0.0804 µmol g^{-1} FW) was produced in the treatment of 0 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin. Regarding embryogenesis, the highest rate was observed in callus subcultures on MS medium supplemented with 1 mg L^{-1} kinetin + 1 mg L^{-1} IBA. The embryogenesis rate positively correlated with increased phenol content. Overall, this study demonstrated successful somatic embryogenesis, callus formation, and enhanced secondary metabolite production through optimized hormonal combinations at novel concentrations in yellow flag, which had not been tested until now. The methods presented in this research are highly valuable for increasing the production of secondary metabolites of this important medicinal and ornamental plant in the pharmaceutical industry.

Abbreviations: Plant Growth Regulators (PGR), 2,4-Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (6-BA), Kinetin (Kin), Indole-3-Butyric Acid (IBA), Indole-3-Acetic Acid (IAA), embryogenic callus (EC), Organ genic callus (OC), non-regenerative callus (NC), Somatic embryos (SE)

Introduction

Iris pseudacorus L., or yellow flag, is a monocotyledonous, rhizomatous perennial plant with distinctive yellow flowers and is a member of the Iridaceae family, which consists of approximately 80 genera and 1,500 species widely distributed across the world (Park et al., 2006). This diploid species (2n = 34), *Iris pseudacorus* L., occurs across a wide variety of climatic and environmental conditions (Beck et al., 2018). Native to Europe, Western Asia, and North Africa, it exhibits high

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adaptability to various aquatic habitats such as wetlands, riversides, and ponds (Kim et al., 2004). It has high tolerance to submersion, variable water pH, and a broad spectrum of soils, and reproduces effectively through rhizomes and water-borne seeds (IUCN, 2022). Due to its striking yellow flowers and ornamental attributes, the plant has been cultivated on every continent except Antarctica (POWO, 2022) and used in ecological restoration initiatives, particularly in wetland environments.

Apart from its ornamental and ecological potential, *I. pseudacorus* L. is a rich source of secondary metabolites such as flavonoids, polyphenolics, anthocyanins, terpenoids, xanthones, and iridals, which possess a variety of pharmacological properties. Extracts from *I. pseudacorus* L. can modulate the differentiation of osteoblasts and osteoclasts (specialized cells involved in bone growth and development) (Kim et al., 2012), and contain compounds responsible for estrogenic activity observed both in vitro and in vivo (Rehab, 2017). Essential oils extracted from rhizomes possess antimicrobial activity against Gram-positive and Gram-negative human pathogenic bacteria (Ramtin et al., 2014). Polyphenols isolated from roots can also inhibit spontaneous colony formation of colon carcinoma HT-29 cells (Tarbeeva et al., 2015). Iridals such as isoiridogermananl and iridobelamal A, isolated from rhizomes, exhibit cytotoxic activity against five human tumor cell lines (Michalak et al., 2021). With increasing demand for bioactive compounds in the pharmaceutical and biotechnology industries, studies on optimizing conditions for the high production of secondary metabolites in this species are of considerable scientific and economic interest (Tarbeeva et al., 2015).

Plant secondary metabolites are known to have a variety of biological activities beneficial to human health. They are becoming increasingly popular due to their unique properties and account for a major portion of the pharmacological, cosmetic, and food industries (Hashim et al., 2021). Irises are primarily utilized as ornamental plants, valued for their colorful flowers, or in the perfume industry for their violet-like fragrance. However, numerous iris species have historically been employed as medicinal plants across various regions, addressing a broad range of ailments. Recent botanical and biochemical studies have expanded our understanding of the chemical constituents in the roots, leaves, and flowers of iris species, highlighting their potential medicinal applications. These investigations reveal that irises are rich in secondary metabolites, with flavonoids and isoflavonoids being the most prevalent. The second most abundant group includes flavones, quinones, and xanthones (Kaššák, 2012).

Obtaining secondary metabolites directly from wild plants has substantial drawbacks, such as requiring long collection periods, posing a risk of species extinction owing to over-exploitation, and yielding limited quantities. To overcome these limitations, there has been a paradigm shift toward the employment of plant tissue culture techniques for the production of key secondary metabolites in vitro. Elicitation appears to be a viable method for increasing phytochemical content and improving the quality of medicinal plants. In vitro culture elicitation activates the plant's defense response and increases the synthesis of secondary metabolites in larger proportions, which are beneficial for therapeutic purposes (Hashim et al., 2021). Different types of plant tissue culture techniques, such as callus culture, somatic embryogenesis, cell suspension culture, hairy root culture, and haploid culture, offer advantages over traditional methods of plant propagation (Kakodkar et al., 2021).

Somatic embryogenesis (SE) is the induction de novo of embryos from somatic plant cells. This morphogenetic pathway can be induced by a number of auxinic herbicides, e.g., 2,4-Dichlorophenoxyacetic acid (2,4-D), Picloram, and Dicamba, at relatively high concentrations (Ramírez-Mosqueda, 2022). In tissue culture, exogenous 2,4-D induces endogenous auxin biosynthesis, which may relate to subsequent auxin independence and somatic embryo induction (Pasternak et al., 2002). However, the precise effects of various concentrations of PGRs on callogenesis, SE, and the biosynthesis of secondary metabolites in *I. pseudacorus* L. remain inadequately explored.

Whereas previous research has established the role of PGRs in callus induction and somatic embryogenesis (with genotype- and hormone combination-dependent results) in various *Iris* species, a substantial research gap exists regarding the specific action of various PGR concentrations on the biosynthesis and accumulation of secondary metabolites in *I. pseudacorus* L. Although in vitro propagation of this species via somatic embryogenesis and polyphenolic compound biosynthesis in callus cultures has been successful (Tarbeeva et al., 2015), and environmental factors such as nutrient levels and culture conditions are known to influence secondary metabolite levels (Mykhailenko et al., 2020), their interaction with PGR treatments in this species has not been investigated in a systematic manner.

Investigations on *I. pseudacorus* L. revealed that explants treated with 4 mg L^{-1} picloram and 1 mg L^{-1} 2,4-D produced the highest callus content (Chamani and Tehri, 2015). Nevertheless, in-depth investigations on the relationship between different PGR concentrations applied during developmental phases and the resulting metabolite profiles in *I. pseudacorus* L. are noticeably absent (Tarbeeva et

al., 2013). Such a knowledge gap prevents the development of optimal protocols for the large-scale production of secondary metabolites from this valuable plant.

The novelty of the present research lies in its examination of a wide spectrum of concentrations of 2,4-D and kinetin, with special emphasis on high concentrations that have been poorly examined in the literature. This study systematically investigates the impacts of these PGRs on callus growth, the process of somatic embryogenesis, and the biosynthesis of secondary metabolites in *I. pseudacorus* L. Studies on other plants have indicated that certain combinations of growth regulators can significantly enhance the production of secondary metabolites, such as in *Taxus brevifolia* and *Dioscorea esculenta* (Karimian et al., 2015; Noor et al., 2024). By providing new information on the relationship between PGRs and metabolic pathways, the present research contributes to both fundamental plant biotechnology and applied pharmaceutical sciences. The findings may enhance growers' and researchers' understanding of how to attain maximum production of bioactive compounds in *I. pseudacorus* L., thereby promoting its medicinal applications and ornamental plant enterprises.

Materials and Methods

Plant materials

This experiment was conducted at the Tissue Culture Technique and Biotechnology Laboratory from 2018 to 2023 to assess callus formation in *Iris pseudacorus* L. using different concentrations of plant growth regulators. *Iris pseudacorus* L. was chosen for this study. This species is native to wetland areas and riverside zones within the lowlands of the Hyrcanian province. Plant rhizomes were collected from the Sari–Juybar road in Mazandaran province at 36˚57′43″ N, 53˚15′63.6″ W. The rhizomes of Yellow Flag (*Iris pseudacorus* L.) were collected from the riverside in August and transported to the laboratory.

Plants were transported in waterproof boxes containing perlite and benomyl fungicide at a concentration of 2 g L^{-1}. After transport, they were maintained under controlled conditions at 16 ± 2 °C with a photoperiod of 16 h light and 8 h darkness for one month to reduce contamination. During the storage period, the substrate was replaced weekly with fresh fungicide solution and deionized water.

Sterilization and explant preparation

The rhizomes were subjected to a sterilization protocol based on previous studies (Abbasi Ghadi et al., 2024). The rhizomes were washed under running water for 1 h after removing the rhizome shell with a scalpel blade. They were then placed in a hot water bath at 46 °C for 60 min. After this, they were agitated for 60 min in a solution containing 2.15 g of the fungicide fluconazole and 0.34 g of the antibiotic streptomycin. Subsequently, they were immersed in 70% alcohol for 1 min, followed by 4% sodium hypochlorite for 10 min. Finally, they were rinsed three times with sterile distilled water and dried with sterile tissue paper.

Tissue culture and proliferation

One rhizome was placed in each glass container filled with commercial MS culture medium (Murashige and Skoog, 1962), supplied by Dochefa Company (The Netherlands). Three weeks after rhizome germination and growth, the explants were transferred to fresh commercial MS medium containing 1.25 mg L^{-1} TDZ and 1.5 mg L^{-1} NAA for proliferation (Abbasi Ghadi et al., 2024). Subsequently, the cultured samples were transferred to a growth chamber at 22 ± 2 °C with a day/night photoperiod of 16/8 h, supplied by 700–800 lux white fluorescent lamps. One month later, the proliferated explants were transferred to a new culture medium to induce callus formation (Table 1). After six weeks, indicators of callus development were assessed. These indicators were survival percentage, callus formation rate, callus fresh weight, callus dry weight, rooting rate, and browning rate.

Table 1. Combination of growth regulator concentrations applied to achieve somatic embryogenesis in yellow flag (L: light and D: dark (absence of light)).

Plant growth regulator															
Growth medium (Light)	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
Growth medium (Dark)	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
2,4-D mg L^{-1}	0	0	0	2.5	2.5	2.5	5	5	5	7.5	7.5	7.5	10	10	10
Kin mg L^{-1}	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1

Measurement of secondary metabolites

Secondary metabolites, including total phenols, flavonoids, and anthocyanins, were measured in callus tissue after the fifth subculture. The flavonoid content of the callus extract was determined using the colorimetric method described by Chang et al. (2010). The total phenolic content was quantified using the Folin–Ciocalteu method (Singleton and Rossi, 1965), while the anthocyanin level was assessed according to Mancinelli's procedure (Mancinelli et al., 1976).

Embryogenesis and organogenesis

At this stage, the callus was transferred from the callogenesis medium (containing 2,4-D and kinetin) to an embryogenesis medium (containing BA, IBA, and kinetin) (Table 2). Various callus types, i.e., embryogenic, organogenic, and non-regenerative (Fig. 1e), were produced during tissue culture on a basal medium supplemented with 2,4-D and kinetin. Embryogenesis was evaluated after eight weeks based on the following parameters: leaf count, maximum leaf length, root count, maximum root length, survival percentage, browning rate, and embryogenesis rate. The survival percentage after eight weeks was recorded as follows:

$$\text{Percentage of explants Survived} = \frac{\sum X\,i}{\text{N}} \times 100$$

Where, Σ = Summation, Xi = Number explants survived, N = Number of explants cultured.

Table 2. Growth regulatory treatments used for embryogenesis on the callus of yellow flag (*Iris pseudacorus* L.).

Treatment number	Plant growth regulator		
	BA (mg L^{-1})	IBA (mg L^{-1})	Kin (mg L^{-1})
1 (control)	0	0	0
2	0.5	1	-
3	1	1	-
4	2	1	-
5	-	1	0.5
6	-	1	1
7	-	1	2

The percentage of browning was calculated by dividing the number of browned callus samples in each treatment by the total number of samples examined in each treatment × 100. Embryogenesis rate was measured by calculating the percentage of explants that formed somatic embryos per explant unit.

Adaptation and growth in the greenhouse

Two months after the samples were placed under embryogenic conditions, the well-grown explants showing clear leaf and root development, particularly around the crown region, were selected and removed from the culture glass containers. The adaptation process for explants derived from embryogenesis began with rinsing them in water to remove residues of the culture medium and agar, thereby minimizing the risk of post-planting soil contamination. After rinsing, the explants were transplanted into small pots containing a 1:1 mixture of coco peat and perlite. They were first placed in a growth room and subsequently transferred to the greenhouse for the adaptation phase (Fig. 1j). The pots were initially covered with plastic to maintain high humidity levels, and the explants were gradually acclimatized by slowly removing the plastic covers. One month after planting, the covers were completely removed, allowing the new plants to continue their growth under standard greenhouse conditions.

Data analysis

In the first experiment, the study was conducted as a factorial experiment based on a completely randomized design with two factors, PGR and light treatment, each with four replications, to examine the influence of two PGRs (2,4-D and Kin) at varying concentrations and different light treatments on callogenesis and secondary metabolite production in Yellow Flag plants. The data were analyzed using SPSS software; mean comparisons were performed via Duncan's multiple range test at the 5% significance level, and the graphs were generated in Excel. In the second experiment, which investigated the effect of PGRs on embryogenesis, the study followed a completely randomized design with seven treatments (Table 2) and three replications. Data analysis was again conducted using SPSS software, with mean comparisons performed using Duncan's multiple range test at the 5% and 1% significance levels, and the graphs were drawn using Microsoft Excel.

Fig. 1. Stages of callus formation and embryogenesis in yellow flag explants grown from callus: (**a**) rhizomes seedling produced by proliferation; (**b**) callus formation; (**c**) yellow callus (nc = non-generative callus); (**d**) green callus; (**e**) the placement of callus in the embryogenesis medium and various types of cell masses (EC = white = embryogenic callus, OC = Green = organogenic callus, NC = Yellow = non-regenerative callus); (**f**) initiation of steps of callus differentiation in different states, such as the heart-shaped state; (**g**) somatic embryos (SE); (**h**) emergence of leaf primordia; (**i**) seedlings embryonated from callus; (**j**) acclimation and cultivation of embryonated seedlings in small pots; (**k**) the growth of seedling shoot in the soil and the initiation of moisture adaptation with the greenhouse environment.

Results

After approximately three weeks of cultivation, the explants in the first treatment (the PGR-free medium) exhibited no response. They remained green and showed no signs of morphogenesis. Based on Duncan's test, the effect of PGRs was statistically significant ($P \leq 0.01$) for all callogenesis parameters, including survival percentage, callus formation rate, callus fresh and dry weight, rooting percentage, and browning percentage (Table 3). The analysis of variance (ANOVA) further demonstrated that the interaction between PGR and light treatment had a significant effect ($P \leq 0.01$) on all callus formation parameters.

Spectrophotometric measurements of secondary metabolites in yellow flag revealed that total phenols, flavonoids, and anthocyanins were significantly affected by PGR concentration. The interactive effect of PGR and light treatment was significant ($P \leq 0.01$) for phenol and flavonoid levels, indicating that these two factors do not act independently but instead exert interdependent influences on metabolite production. However, the interaction effect was not significant for anthocyanin levels; in this case, only the simple effects of light treatment and PGRs were significant ($P \leq 0.01$).

Callus formation parameters

Maximum callogenesis rate (100%) (Table 4) was achieved in response to treatments L12 (7.5 2,4-D + 1 Kin), L13 (10 2,4-D + 0 Kin), L14 (10 2,4-D + 0.5 Kin), and L15 (10 2,4-D + 1 Kin). No callus formation was observed in the PGR-free basic culture medium (control) in treatments L1 (0 2,4-D

+ 0 Kin), L2 (0 2,4-D + 0.5 Kin), D1 (0 2,4-D + 0 Kin), and D2 (0 2,4-D + 0.5 Kin). In these treatments, the explants survived by increasing leaf and root growth when placed in the culture medium. Overall, light treatment and PGR application had a pronounced effect on the rate of callus formation (Fig. 2).

Table 3. Analysis of variance (ANOVA) for light and hormonal treatment effects and their interaction on callogenesis and secondary metabolite production.

S.O.V	df	Phenol	Flavonoid	Anthocyanin	Survival %	Callus formation	Fresh callus weight	Dry callus weight	Rooting %	Browning %
Light Treatment	1	800.73**	4331.16**	0.001**	1683.75**	3015.01**	2.57**	2.81**	5852.03**	1404.25**
(PGR)	14	653.92**	1878.28**	0.003**	343.39**	13001.95**	4.04**	3.80**	9836.41**	330.42**
Light Treatment×(PGR)	29	85.13**	209.42**	0.00004ns	272.93**	551.91**	0.49**	0.48**	1006.39**	265.48**
Error	90	4.79	6.26	0.00003	1.68	3.45	0.001	0.003	4.37	2.04
CV (%)	_	12.90	6.73	26.01	1.36	2.99	3.88	5.35	8.15	32.44

**, and ns represent significant differences at the 1% and the lack of significant differences, respectively.

Fig. 2. Comparison of callus formation rate in (**A**) light and (**B**) dark treatments.

Survival percentage

The comparison of means revealed that (Table 4) the highest survival percentage (100%) was observed in treatments L9 (5 2,4-D + 1 Kin), L11 (7.5 2,4-D + 0.5 Kin), L12 (7.5 2,4-D + 1 Kin), L13 (10 2,4-D + 0 Kin), L15 (10 2,4-D + 1 Kin), D1 (0 2,4-D + 0 Kin), D5 (2.5 2,4-D + 0.5 Kin), D6 (2.5 2,4-D + 1 Kin), D8 (5 2,4-D + 0.5 Kin), D9 (5 2,4-D + 1 Kin), D11 (7.5 2,4-D + 0.5 Kin), D13 (10 2,4-D + 0 Kin), and D15 (10 2,4-D + 1 Kin). The lowest survival percentage was 57.5%, recorded by treatment L3 (0 2,4-D + 1 Kin).

Comparison of means for the interactive effect of PGRs and light treatments

Based on the comparison of the interaction with light treatment and PGR, the average Survival percentage was higher in dark conditions than in light conditions.

Callus fresh and dry weight

Regarding callus fresh weight, the highest value was 2.47 g, recorded in treatment D15. Excluding the PGR-free control treatments L1 and L2, where no callus was formed, the lowest callus fresh weights were observed in treatments D3 (0.007 g) and D4 (0.03 g), both of which contained the lowest concentrations of 2,4-D and kinetin. The findings indicated that both the highest and lowest callus fresh weights were obtained under dark conditions. The highest callus dry weight was 2.40 g, obtained from treatment D15 (10 2,4-D + 1 Kin), while the lowest was 0.003 g, associated with treatment D3 (Table 4).

Table 4. Comparison of means for the interactive effect of PGR and light treatment on secondary metabolites and callus formation parameters.

Light treatment (a) × PGRs (b)	Phenol (mg 100 g^{-1})	Flavonoid (mg 100 g^{-1})	Anthocyanin (µmol g^{-1} FW)	Survival rate %	Callus formation %	Fresh callus weight (g)	Dry callus weight (g)	Rooting %	Browning %
L1	2.32^{q}	17.11^{n}	0.024^{d-g}	97.25^{bc}	0^{l}	0^{q}	0^{x}	100^{a}	2.75^{ghi}
L2	5.68^{p}	43.52^{ef}	0.022^{efg}	97.75^{abc}	0^{l}	0^{q}	0^{x}	100^{a}	2.25^{g-j}
L3	7.43^{op}	57.13^{c}	0.085^{a}	57.5^{i}	2.5^{kl}	0.32^{p}	0.28^{w}	96^{b}	42.5^{a}
L4	7.60^{n-p}	41.98^{fg}	0.032^{d}	75.5^{h}	14.5^{j}	0.47^{o}	0.405^{q}	58.25^{d}	22.75^{b}
L5	10.45^{k-o}	72.09^{a}	0.005^{j}	80.75^{g}	48.62^{g}	0.85^{m}	0.795^{o}	59^{d}	19.25^{c}
L6	11.01^{j-n}	38.85^{gh}	0.012h^{ij}	93^{e}	44.25^{h}	0.93^{l}	0.842^{no}	32.5^{e}	7^{e}
L7	14.86^{f-i}	46.97^{e}	0.017^{ghi}	98.75 ^{ab}	52.5^{f}	1.027^{k}	0.945^{m}	22.75^{fg}	1.25^{hij}
L8	14.10^{f-j}	50.81^{d}	0.007^{j}	99.37^{a}	70.75^{d}	0.962^{l}	0.892^{mn}	8^{h}	0.625^{ij}
L9	27.49^{c}	51.20^{d}	0.010^{ij}	100^{a}	64.5^{e}	1.182^{j}	1.102^{l}	9.75 ^{h}	0^{j}
L10	42.42^{b}	67.13^{b}	0.027^{def}	84.5^{f}	71.5^{d}	1.182^{j}	1.127^{kl}	1.75^{i}	14^{d}
L11	22.05^{de}	33.62^{ij}	0.006^{j}	100^{a}	86.5^{c}	1.547^{g}	1.5^{g}	1.25^{i}	0^{j}
L12	17.04^{f}	30.10^{jkl}	0.010^{ij}	100^{a}	100^{a}	1.647^{f}	1.572^{fg}	0^{i}	0^{j}
L13	13.31^{g-k}	31.51^{jk}	0.028^{de}	100^{a}	100^{a}	1.345^{i}	1.287^{i}	0^{i}	0^{j}
L14	11.27^{j-m}	37.09^{hi}	0.031^{d}	95.25^{d}	100^{a}	1.397^{i}	1.265^{ij}	0^{i}	5^{ef}
L15	8.60^{m-p}	29.06^{kl}	0.048^{c}	100^{a}	100^{a}	1.24^{j}	1.2^{jk}	0^{i}	0^{j}
D1	9.01^{l-p}	13.78^{no}	0.017^{ghi}	100^{a}	0^{l}	0^{q}	0^{x}	100^{a}	0^{j}
D2	22.44^{de}	22.63^{m}	0.019^{fgh}	98^{abc}	0^{l}	0^{q}	0^{x}	100^{a}	2^{g-j}
D3	11.47^{i-m}	42.29^{fg}	0.076^{b}	98a^{bc}	1.75 ^{l}	0.007^{q}	0.003^{x}	25.5^{f}	2^{g-j}
D4	22.41^{de}	32.73^{jk}	0.026^{d-g}	96^{cd}	5^{k}	0.035^{q}	0.018^{x}	20.75^{g}	4^{fg}
D5	21.16^{e}	63.64^{b}	0.003^{j}	100^{a}	41^{i}	0.51^{o}	0.467^{q}	9.5^{h}	0^{j}
D6	16.39^{fg}	26.80^{l}	0.008^{ij}	100^{a}	64.5^{e}	0.765^{n}	0.695^{p}	3.25^{h}	0^{j}
D7	23.99^{de}	41.89^{fg}	0.010^{ij}	100^{a}	93.75^{b}	1.345^{i}	1.29^{i}	7.25^{h}	0.25^{j}
D8	16.40^{fg}	30.69^{jk}	0.004^{j}	100^{a}	100^{a}	1.462^{h}	1.397^{h}	6.75^{h}	0^{j}
D9	16.51^{fg}	31.01^{jk}	0.005^{j}	100^{a}	100^{a}	1.69^{ef}	1.635^{ef}	3^{i}	0^{h}
D10	47.95^{a}	55.56^{c}	0.006^{j}	100^{a}	100^{a}	1.75^{e}	1.687^{e}	2.7^{i}	3^{fgh}
D11	25.33^{cd}	11.89^{o}	0.003^{j}	100^{a}	100^{a}	1.815^{d}	1.767^{d}	1^{i}	0^{j}
D12	16.37^{fg}	14.52^{no}	0.008^{ij}	100^{a}	100^{a}	2.145^{c}	2.057^{c}	0^{i}	2.2^{g-j}
D13	15.29^{fgh}	14.20^{no}	0.021^{efg}	100^{a}	100^{a}	2.325^{b}	2.275^{b}	0^{i}	0^{j}
D14	15.94^{fg}	55.54^{c}	0.024^{d-g}	100^{a}	100^{a}	2.175^{c}	2.11^{c}	0^{i}	1.25^{hij}
D15	12.45^{h-l}	10.76^{o}	0.044^{c}	100^{a}	100^{a}	2.475^{a}	2.405^{a}	0^{i}	0^{j}

Different letters in each column differed significantly based on Duncan's multiple range test.

Rooting percentage

The results regarding rooting percentage revealed that yellow flag exhibited the highest rooting rate (100%) when placed in media free of PGRs (L1, D1) or containing low PGR concentrations (L2, D2) under both light and dark conditions. However, as the concentrations of 2,4-D and kinetin increased, the rooting percentage declined, callus production increased, and both growth rate and root development diminished proportionally, leading to increased callus weight and size. The lowest rooting rate (0%) was observed in treatments ranging from L12 to L15 and from D12 to D15.

Browning percentage

The comparison of means for the interactive effect on browning percentage revealed that most treatments resulted in minimal browning, and the majority of samples exhibited healthy growth. The extent of browning in Yellow Flag callus indicated that the highest browning (42.5%) occurred in treatment L3 (0 2,4-D + 1 Kin), while no browning was observed under light conditions in treatments L9, L11, L12, L13, L15, D1, D5, D6, D8, D9, D11, D13, and D15.

Comparison of means for the interactive effect of PGRs and light treatments on secondary metabolites of induced callus

The interactive effect of PGRs and light treatment was significant ($P \leq 0.01$) for total phenol and flavonoid content. However, this interaction did not significantly affect anthocyanin levels (Fig. 3); nevertheless, the simple effects of PGRs and light treatment on anthocyanin were significant ($P \leq 0.01$) (Table 3). The comparison of means among treatments revealed that the highest total phenol content (47.95 mg 100 g^{-1}) was associated with treatment D10 (7.5 2,4-D + 0 Kin), while the lowest content (2.32 mg 100 g^{-1}) occurred in treatment L1, the PGR-free control. These data suggest that the application of PGRs contributes to increasing phenolic compound production in yellow flag plants.

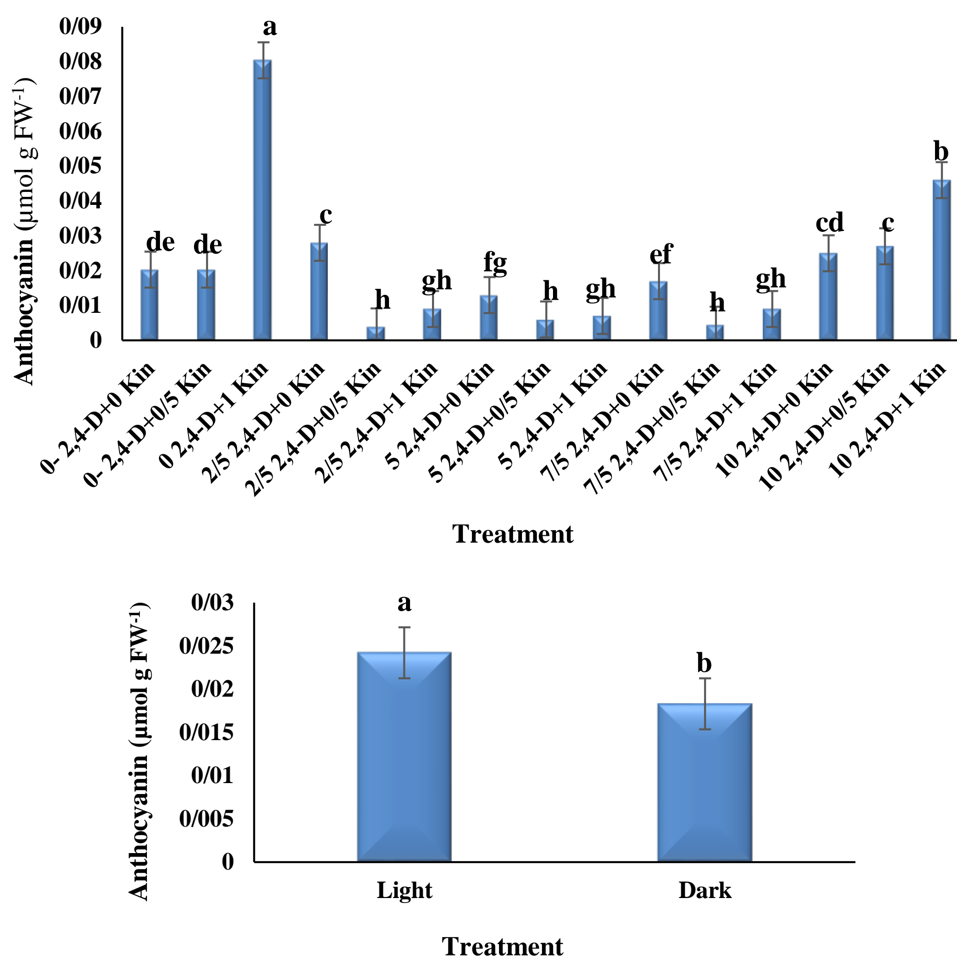

Fig. 3. Comparison of means for the simple effects of PGRs (top) and light treatments (down) on anthocyanin synthesis by yellow flag. (Microsoft Excel 2013).

Nonetheless, a comparison of total phenol production under dark and light conditions within similar PGR treatments showed that phenol production was consistently higher in darkness than in light. The comparison of means for flavonoid content indicated that the highest value (72.08 mg 100 g^{-1}) occurred in treatment L5 (2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin), while the lowest value (10.75 mg 100 g^{-1}) was observed in treatment D15 (10 2,4-D + 1 Kin), which contained the highest concentrations of both 2,4-D and kinetin. This pattern suggests that high concentrations of the two PGRs negatively affect flavonoid production under dark conditions. Comparisons of flavonoid production in light and dark conditions further revealed that darkness adversely affected flavonoid levels, whereas light conditions in the same treatments resulted in higher flavonoid accumulation in the callus of yellow flag grown in media containing 2,4-D and kinetin. In other treatments, increasing the concentrations of 2,4-D and kinetin led to an increase in flavonoid content relative to the control. However, further increases in PGR concentration eventually exerted a detrimental effect on flavonoid production, as demonstrated by its declining trend from treatment L10 to treatment L15.

The comparison of means for the simple effects of PGRs showed that the highest anthocyanin content (0.0804 µmol g^{-1} FW) in Yellow Flag corresponded to the treatment 0 2,4-D + 1 Kin, in which kinetin was applied at 1 mg L^{-1} and no 2,4-D was used. This finding suggests that kinetin enhances anthocyanin production, whereas 2,4-D not only fails to contribute positively but also reduces anthocyanin levels in yellow flag. The lowest anthocyanin content (0.0045 µmol g^{-1} FW) was obtained from the treatment 7.5 2,4-D + 0.5 Kin.

The results concerning the simple effects under different light conditions showed that anthocyanin content was significantly greater in light (0.0242 µmol g^{-1} FW) than in darkness (0.0183 µmol g^{-1} FW), indicating that light exposure plays a positive role in enhancing anthocyanin synthesis in yellow flag.

Correlation of callus formation parameters and secondary metabolites under light and dark conditions

The results of correlations between metabolic traits and callus formation revealed that the browning percentage exhibited significant correlations with flavonoid content (P ≤ 0.05), anthocyanin content (P ≤ 0.01), survival percentage (P ≤ 0.01), callus formation rate (P ≤ 0.05), and rooting percentage (P ≤ 0.05). An inverse relationship was observed between the callus formation rate and survival percentage on one hand, and the browning percentage on the other, indicating that as callus formation and survival percentages increased, the browning percentage correspondingly decreased. With respect to the browning percentage index, an increase in survival percentage was consistently associated with a proportional decrease in browning percentage (Table 5).

Table 5. Simple correlation analysis of callus-related traits and secondary metabolites under PGR treatments in light conditions in *Iris pseudacorus* L.

	1	2	3	4	5	6	7	8	9
1. Phenol (mg 100 g^{-1})	1								
2. Flavonoid (mg g^{-1})	0.288	1							
3. Anthocyanin (µmol g^{-1} FW)	-0.35	0.048	1						
4. Survival percentage (%)	0.18	-0.441*	-0.519**	1					
5. Callus formation (%)	0.345	-0.125	-0.357	0.408*	1				
6. Callus fresh weight (g)	0.301	-0.188	-0.27	0.312	0.926**	1			
7. Callus dry weight (g)	0.303	-0.199	-0.266	0.32	0.922**	0.999**	1		
8. Rooting rate (%)	-0.443*	-0.006	0.266	-0.444*	-.847**	-0.757**	-0.750**	1	
9. Browning rate (%)	-0.158	0.449*	0.516**	-0.996**	-.388*	-0.287	-0.295	0.437*	1

ns, *, and ** represent non-significance and significance at the $P \leq 0.05$ and $P \leq 0.01$ levels, respectively.

The rooting percentage displayed a negative correlation with phenol content, survival percentage, callus formation rate, and both the fresh and dry weights of the callus. This suggests that an increase in rooting percentage leads to a decrease in phenol content, and conversely, increases in callus formation and in the fresh and dry weights of the callus are associated with reduced rooting percentage. Callus dry weight was positively correlated with both callus formation rate and callus fresh weight (P ≤ 0.01). Callus fresh weight exhibited a positive correlation only with the callus formation rate (P ≤ 0.01). A positive correlation was also observed between callus formation percentage and survival percentage (P ≤ 0.05). In contrast, the survival percentage index demonstrated a negative correlation with flavonoid content (P ≤ 0.05) and anthocyanin content (P ≤ 0.01).

Embryogenesis from the callus grown

Four weeks after cultivation, the callus began to undergo visible changes, because much of the initially dense, yellow callus gradually turned green (Fig. 4e). During the early stages of embryogenesis, some callus masses developed yellow-green, spherical structures characteristic of embryogenic initiation. By six weeks after cultivation, clear signs of embryogenesis were evident across the treatments. The ANOVA for embryogenesis-related traits of yellow flag under a 16/8 h light/dark photoperiod across three replications (Table 6) indicated significant differences in the number of shoots, length of the longest shoot, embryogenesis rate, browning percentage, and survival percentage (P < 0.01), as well as in root count (P < 0.01).

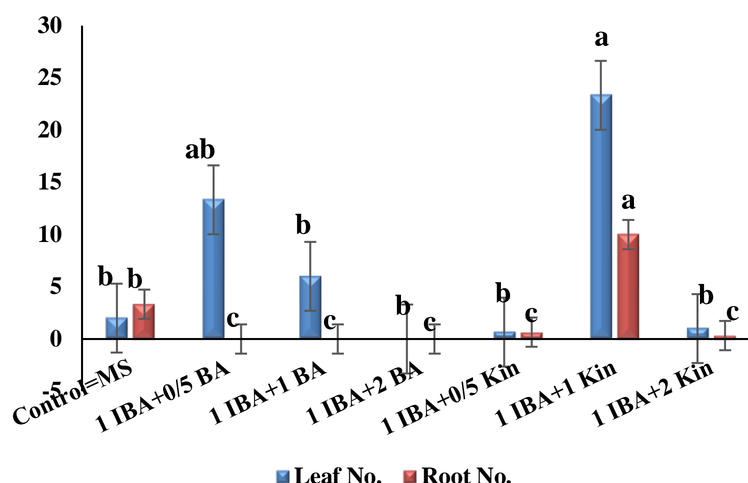

Fig. 4. Comparison of means for leaf and root count in different embryogenesis treatments of yellow flag. (Microsoft Excel 2013).

Table 6. Analysis of variance (ANOVA) for somatic embryogenesis traits derived from callus in yellow flag.

SOV	df	Leaf No.	Length of the longest leaf (cm)	Root No.	Length of the longest root (cm)	Organogenesis rate (%)	Browning rate (%)	Survival rate (%)
PGR	6	228.49*	3.59*	41.15**	1.099^{ns}	2966.27*	3035.71*	3035.71*
Error	14	61.85	1.158	2.14	0.393	773.81	922.62	922.62
CV	-	28.3	8.6	17.49	15.8	10.15	4.39	9.81

**, *, and ^{ns} represent significance at the $P < 0.01$ and $P < 0.05$ levels, and non-significance, respectively.

However, the length of the longest root did not show any significant differences across the various PGR treatments for embryogenesis. In other words, ANOVA revealed significant differences among the embryogenesis treatments for seven of the assessed traits. The comparison of means (Table 7) for leaf-related traits showed that the treatment containing 1 mg L^{-1} IBA + 1 mg L^{-1} Kin produced the highest number of leaves, with an average of 23.33 leaves (Fig. 1), as well as the greatest average leaf length (2.94 cm). Additionally, this treatment yielded the highest root count, with an average of 10 roots. This outcome may be attributed to the influential role of leaves in promoting rooting, as leaves contain substantial amounts of auxins, a class of PGRs essential for stimulating root growth. The application of exogenous auxin, together with an increased number of leaves and their proportional distribution on the plant body, facilitates the initiation of root development from embryos (Chuengpanya et al., 2022).

Table 7. Comparison of means for the morphological traits of embryogenesis using Duncan's multiple-range test.

	Leaf No.	Length of the longest leaf (cm)	Root No.	Length of the longest root (cm)	Organogenesis rate (%)	Browning rate (%)	Survival rate (%)
Control (MS)	2^{b}	0.4^{b}	3.33^{b}	1.1^{ab}	16.66^{b}	25^{b}	75^{a}
1 IBA + 0.5 BA	13.33^{ab}	1.99^{ab}	0^{c}	0^{b}	41.66^{b}	50^{b}	50^{ab}
1 IBA + 1 BA	6^{b}	1.14^{ab}	0^{c}	0^{b}	25^{b}	33.33^{ab}	66.66^{a}
1 IBA + 2 BA	0^{b}	0^{b}	0^{c}	0^{b}	0^{b}	0^{b}	100^{a}
1 IBA + 0.5 Kin	0.66^{b}	0.31^{b}	0.66^{c}	0.13^{b}	8.33^{b}	91.66^{a}	25^{b}
1 IBA + 1 Kin	23.33^{a}	2.94^{a}	10^{a}	1.43^{a}	91.66^{a}	8.33^{b}	91.66^{a}
1 IBA + 2 Kin	1^{b}	0.25^{b}	0.33^{c}	0.09^{b}	8.33^{b}	91.66^{a}	8.33^{b}

Furthermore, the highest rate of organogenesis (91.66%) was observed in the same treatment. The most extensive browning (91.66%) occurred in response to treatments 1 mg L^{-1} IBA + 0.5 mg L^{-1} Kin or 2 mg L^{-1} Kin. The highest survival percentage (100%) was associated with 1 mg L^{-1} IBA + 1 mg L^{-1} BA. It can be concluded that the callus of the yellow flag, when grown in environments with IBA + BA, did not produce roots despite undergoing embryogenesis and branching. In contrast, in environments with IBA + Kin, they successfully produced leaves and roots.

Discussion

Callogenesis is influenced by several factors, including the type of explant and the concentration and composition of PGRs in the culture medium (Ali et al., 2017). The culture medium type, the strength of the salts in the culture medium, and the type and concentration of PGRs used are crucial determinants for successful tissue culture outcomes (Fargoso Monfort et al., 2018). Varied responses in callogenesis and morphology can be attributed to the role of PGR performance in managing the cell cycle. PGRs regulate essential enzymes involved in the cell cycle, and their exogenous application modifies endogenous PGR levels, leading to altered growth patterns and plant cell wall modifications (Ashokhan et al., 2020). In fact, in vitro growth and morphogenesis are affected by the interplay and proportion of exogenously applied PGRs and those produced endogenously by cultured cells (Zulkarnain, 2009).

In European yew trees, various ratios of 2,4-D have been utilized for callogenesis. Indeed, hormone combinations have been proven to be more effective than individual applications (Abbasi Kajani et al., 2014). In a study on thyme (*Thymus kotschyanus*), researchers obtained the highest callus fresh weight from MS medium containing 2 mg L^{-1} of 2,4-D and 1 mg L^{-1} of kinetin (Mosavat et al., 2019). Karamian and Ranjbar (2010) reported that high concentrations of kinetin inhibited callus growth and fresh weight, whereas 2,4-D linearly stimulated callus growth and fresh weight. They stated that higher concentrations of 2,4-D, up to 2 mg L^{-1} or even beyond, would give better results than lower concentrations (Karamian and Ranjbar, 2010). Optimal callus growth has reportedly been associated with 2,4-D concentrations of 2, 3, and 5 mg L^{-1} (Bose et al., 2016).

In a study on the tissue culture and callus formation of *Verbascum scamandri* Murb., researchers applied 2,4-D and kinetin at different rates and observed that callus formation reached 100% with increasing rates of both PGRs (Cambaz and Çördük, 2023). Thus, a combination of auxin and cytokinin appears to be more effective for initiating callus formation, seemingly due to their stimulatory and regulatory effects on cell division. Research has shown that the combined use of cytokinin and auxin is more effective than their singular application (Shirin et al., 2007). The current research on yellow flag agrees with previous studies, indicating that higher concentrations of 2,4-D (7.5–10 mg L^{-1}) enhanced callus fresh weight. The addition of cytokinin at low rates to the MS medium containing 2,4-D increased callus growth rate and weight. It can be concluded that the source, age, and endogenous phytohormone levels in the explant, along with the type, concentration, and mode of action of the applied auxins and cytokinins, may be critical for callus formation (Qahtan et al., 2022).

Hosseini et al. (2015) reported that the highest callogenesis in various citrus species was achieved when 2,4-D was applied at 2 or 3 mg L^{-1} and kinetin (Kin) at 3 or 6 mg L^{-1}. In another study, researchers achieved 93% callogenesis in *Kelussia odoratissima* Mozaff. by applying 2 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} of Kin (Razeghi et al., 2015). These results contradict those of Soreni et al. (2012), who reported the best callus percentage of *C. cyminum* in the treatment containing 1 mg L^{-1} of 2,4-D. Callogenesis is a much more challenging process in monocotyledons, particularly in the iris family, than in other monocot families such as Amaryllidaceae (Liu, 2020). Vahedi et al. (2015) successfully achieved callus formation in saffron by applying a combination of 2,4-D and kinetin, which is consistent with the reports of Chaloushi et al. (2007), Karamian and Ranjbar (2010), and Dalila et al. (2013). They observed the highest frequency of callogenesis from the terminal meristem in MS medium containing 2 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} of kinetin.

Some researchers argue that 2,4-D is necessary for the formation of morphogenic calluses in the callus culture of *I. pumila*, *I. pseudacorus*, and *I. virginica*. Boltenkov et al. (2007) revealed that the capacity for callus formation in *I. ensata* was influenced by the PGR content in the induction culture medium. They obtained the highest callus frequency (over 90%) from media containing 1–2 mg L^{-1} of 2,4-D and 0.2–0.5 mg L^{-1} of kinetin. These two hormones were effective in the proliferation and organogenesis of this iris species. These results agree with ours regarding the usefulness of 2,4-D and kinetin in inducing callus formation. Boltenkov (2011) explored the effect of PGRs on callus tissue induction in immature embryos of yellow flag and found that the addition of 2,4-D and kinetin to MS medium was necessary for callogenesis and callus formation in this species.

In a study on callus formation in the endangered iris species *Iris dichotoma* Pall., Bae (2021) reported that calluses had their best growth in the presence of 1 mg L^{-1} of 2,4-D, demonstrating the key role of this PGR in iris callus proliferation. In this regard, he showed

that 2,4-D not only induced callus formation but also made callus proliferation possible, which corroborates previous results observed in the callus culture of yellow flag iris (Boltenkov et al., 2004). Our results regarding the positive effect of high concentrations of 2,4-D in callogenesis, along with kinetin, are supported by other researchers.

The mass of callus generated in tissue culture is primarily determined by the rate of cell division and proliferation, which is followed by cell enlargement. The rate of cell division may be influenced by specific cytokinin and auxin compounds at particular concentrations, depending on the plant species and external conditions such as light intensity and ambient temperature. Among PGRs, 2,4-D is pivotal in facilitating cell division and elongation and in regulating cell growth (Santos et al., 2014). Research into the impact of varying concentrations of 2,4-D and kinetin on yew plants revealed that both PGRs enhanced callus fresh weight and that 2,4-D notably induced callus growth.

Farvardin et al. (2017) conducted a study on cumin plants using different concentrations of 2,4-D (1.5, 2, 2.5, 3, and 3.5 mg L^{-1}) and kinetin (0, 0.2, 0.5, and 1 mg L^{-1}). The findings indicated that the culture medium treated with 2,4-D and kinetin significantly influenced callus formation. In addition, there were significant differences among the concentrations of growth regulators in relation to the generated callus weight. The highest response in callus formation (100%) was achieved at 2,4-D concentrations of 2 and 2.5 mg L^{-1} and kinetin concentrations of 0, 0.2, 0.5, and 1 mg L^{-1} in both indigenous cumin landraces.

Regarding callus color and texture, it is believed that incorporating auxin into the culture medium positively influences callus colour and texture during both the callogenesis and proliferation stages. Previous studies have indicated that adding varying concentrations of 2,4-D to the culture medium leads to the production of callus that are brittle and may appear green or yellow (Najhah et al., 2021). In our current research, the addition of 2,4-D to the culture medium resulted in the formation of green and yellow callus under light conditions and yellow and cream callus in dark conditions. According to Putri (2008), light plays a significant role in influencing cell metabolism and the effectiveness of PGRs in the culture medium. Tissue culture in darkness facilitates the functioning of auxin, thereby expediting the callus formation process. Chawla (2009) emphasized the critical role of light in tissue culture, particularly its impact on callus formation and photomorphogenesis. The status of a plant in tissue culture is significantly affected by such factors as the photoperiod, light quality, and intensity (Siregar et al., 2010). Research indicates that low light intensity is crucial for effective callogenesis, particularly when combined with specific auxins. For example, the interaction of auxin and light conditions in callus formation of *Iris* sp. is a complex interplay that significantly influences plant regeneration. The balance of auxin and light conditions is critical, as light can modify auxin transport and signalling pathways, influencing overall plant development (Tian and Reed, 2001). In contrast, higher light levels can inhibit callus growth, suggesting that light intensity directly affects auxin efficacy in callogenesis (Laublin et al., 1991). Chen et al. (2019) demonstrated that light intensity was a crucial factor for achieving optimal callus formation. The findings revealed that a light intensity of 10 µmol m^{-1} s^{-1} was more conducive to callus proliferation than 45 µmol m^{-1} s^{-1} across a broad range of PGR compounds. Light stimulates callogenesis by activating enzymes involved in cell division, one of which is the cyclin-dependent kinase (CDK) enzyme during the terminal S, M, and G phases of the cell cycle. Darkness aids in callus induction by supporting auxin activity, as light exposure can degrade auxin and redirect its transport toward light-avoiding organs (Anatushshoimah et al., 2020). Indeed, the incorporation of auxin into the environment prompts cell division and enlarges explants, thereby stimulating callus formation and growth (Rahayu et al., 2003). The comparison of callus formation under the light treatments in this study indicated a higher rate of callus formation in dark conditions than in light conditions.

Jamwal et al. (2018) found that PGRs could influence the synthesis rates of secondary metabolites. According to Fargoso Monfort et al. (2018), the optimal concentration of nutrients is crucial for the growth of explants and the accumulation of secondary metabolites. In most cases, the PGR concentration plays a pivotal role in the accumulation of secondary metabolites (Mantell and Smith, 1984). Cytokinin type and concentration, as well as the ratio of auxin to cytokinin, significantly affect the growth of plant cells (Mok et al., 1976). Cytokinins exert diverse effects depending on the metabolic processes and plant species involved. For instance, the kinetin application has been shown to enhance anthocyanin production in *Haplopappus gracilus*, like the present work in which the highest anthocyanin levels were achieved at the higher kinetin concentration. Conversely, kinetin has been observed to inhibit anthocyanin production in *Populus* sp. (Meyer and Van Staden, 1995). The application of 2,4-D has been reported to stimulate carotenoid production in carrot suspension cultures and anthocyanin production in *Oxalis linearis* callus cultures (Chamani et al., 2017; Chuengpanya et al., 2022).

Substituting 2,4-D with an alternative auxin is essential for initiating morphogenesis in the Yellow Flag. The phytohormones of indole acetic acid (IAA) (2 mg L^{-1}) and kinetin (1 mg L^{-1}) induced

embryogenesis in this species and a notable acceleration in primary root growth was observed in media containing IBA (0.5 mg L^{-1}) (Boltenkov, 2011). These findings are consistent with the current research, which corroborates the beneficial impact of IBA and Kin on embryogenesis in yellow flag callus tissue. Regarding PGRs involved in differentiation in culture media, BA is the key to branch embryogenesis *in vitro*. However, an increase in BA concentration was linked to a reduction in callus growth and an increase in browning. Higher concentrations of BA in the culture medium, beyond the optimal level, have been shown to diminish the emergence of new shoots in two iris species, *Iris collettii* Hook. f. and *I. domestica* (L.) Goldblatt and Mabb (Shibli and Ajlouni, 2000). Comparable outcomes were noted in the number of shoots derived from embryogenic callus in *I. nigricans* (Nasircilar et al., 2010). Also, *I. odaesanensis* (Bea et al., 2021) and *I. stenophylla* (Ayabe and Sumi, 1998), when subjected to BA concentrations higher than optimal, exhibited symptoms of callus browning and loss. These observations align with our finding that increasing BA concentration to 2 mg L^{-1} alongside 1 mg L^{-1} IBA led to callus browning and mortality in the embryogenesis medium (Fig. 5).

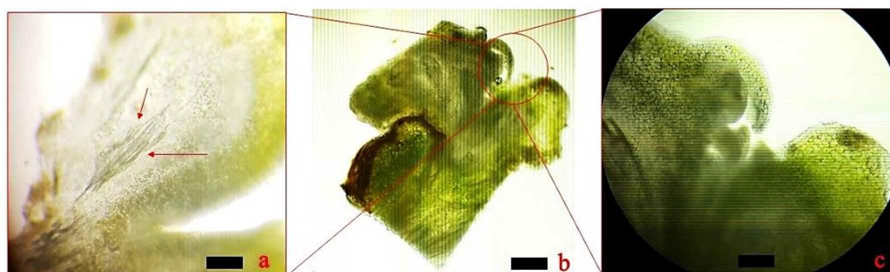

Fig. 5. (**a**) The emergence of initial vascular tissue; (**b**) the cross-section of the callus placed in the embryogenesis medium; (**c**) the emergence of leaf primordia.

In a study on *I. koreana* embryogenesis, 1.0 or 3.0 mg L^{-1} of BA induced complete shoot embryogenesis. However, its highest concentration, i.e., 3.0 mg L^{-1}, seemed to inhibit shoot growth (Bea et al., 2021). BA is reportedly crucial for shoot formation from embryos *in vitro* (Liu et al., 2020; Myers and Simon, 1999). Our results concur with those of Myers and Simon (1999) who found that BA alone was insufficient for embryogenesis. This result is consistent with Luciani and colleagues, who showed that BA causes branching in embryos derived from callus (Luciani et al., 2006). The combination of a PGR like IBA with BA was effective in inducing embryogenesis in yellow flag callus, consistent with the findings of the researchers mentioned above. In *I. koreana*, callus derived from shoots were placed in a 1.2 MS culture medium with varying IBA concentrations (0, 0.5, 1, and 3 mg L^{-1}) to study root induction. After four weeks, over 90% of the seedlings exhibited root induction, with the longest roots appearing in the 1.2 MS medium with 1 mg L^{-1} IBA (Bea et al., 2021). Although Bae et al. (2021) used IBA and BA in separate stages to induce root and shoot development in *I. koreana*, the ideal IBA concentration for maximizing root count and length was determined to be 1 mg L^{-1}, which is similar to our findings.

Conclusion

This study investigated the effects of plant growth regulators (PGRs) and light treatments on callogenesis, secondary metabolite production, and somatic embryogenesis in *Iris pseudacorus* L. (yellow flag). The results demonstrated that PGR-free media failed to induce morphogenesis, with explants remaining green but continuing their vegetative growth. The results of tissue culture in the callogenesis medium indicated that the type and low to high concentrations of PGRs in the MS medium (2,4-D and kinetin) and the light conditions significantly influenced callus development and the synthesis of phenols, flavonoids, and anthocyanins. Overall, based on comparing PGR and light treatments, the highest phenol content was recorded under the PGR treatment of 7.5 mg L^{-1} 2,4-D + 0 mg L^{-1} Kin, whereas, regarding light conditions, phenol content was higher in darkness than in light. The accumulation of phenolic compounds in plants is influenced by two environmental factors: light and temperature. Light perception is mediated by specialized photoreceptors, which induce transcriptional modifications that regulate the biosynthesis of phenolic compounds (Volna et al., 2024).

Flavonoid levels peaked in the fifth (L5) and tenth (L10) treatments, being notably higher under light than in darkness. Although anthocyanin production was not significantly affected by the interaction of the treatments, it was higher in light conditions than in dark conditions under similar PGR treatments. Analysis of the simple effect of PGRs revealed that the maximum production of anthocyanins was achieved with the treatment of 0 mg L^{-1} 2,4-D + 1

mg L^{-1} Kin. This underscores the beneficial impact of the highest kinetin concentration (1 mg L^{-1}) and the inhibitory effect of 2,4-D on anthocyanin synthesis. The greatest callus formation occurred in dark conditions under treatments D10–D15, while in light conditions it was obtained from treatments L12–L15, highlighting the more pronounced effect of darkness on callus development. The heaviest callus fresh and dry weights in dark conditions were associated with the treatment containing the highest concentrations of both PGRs, while the lowest weights corresponded to the treatment of 0 mg L^{-1} 2,4-D + 1 mg L^{-1} Kin. Under light conditions, the maximum callus fresh and dry weights were observed in treatment L12 (7.5 mg L^{-1} 2,4-D + 1 mg L^{-1} Kin).

Regarding embryogenesis rates, callus explants in MS medium containing 1 mg L^{-1} IBA + 1 mg L^{-1} Kin outperformed all other tested combinations and concentrations. The findings suggest that employing callus and inducing somatic embryogenesis with optimal PGR combinations enhances callus formation and boosts the production of secondary metabolites in the yellow flag iris. This study evaluated the impact of plant growth regulators (PGRs) and light treatments on callogenesis, secondary metabolite production, and somatic embryogenesis in *Iris pseudacorus* L. PGR-free media failed to induce morphogenesis, while 7.5–10 mg L^{-1} 2,4-D with 0–1 mg L^{-1} kinetin under dark conditions achieved maximum callogenesis (100%), fresh weight (2.47 g), and dry weight (2.40 g). Secondary metabolite production was optimized with 7.5 mg L^{-1} 2,4-D (no kinetin) for phenols (47.95 mg 100 g^{-1}), 2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin for flavonoids (72.08 mg g^{-1}), and 0 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin for anthocyanins (0.0804 µmol g^{-1} FW), with darkness favoring phenols and light enhancing flavonoids and anthocyanins. Somatic embryogenesis was most effective with 1 mg L^{-1} IBA + 1 mg L^{-1} kinetin, yielding 91.66% organogenesis, 23.33 leaves, and 10 roots, with minimal browning (8.33%). Optimal treatments included: a2b10 (7.5 mg L^{-1} 2,4-D, dark) for phenols; a1b5 (2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin, light) for flavonoids; a1b3 (0 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin, light) for anthocyanins; a1b12–a1b15 (7.5–10 mg L^{-1} 2,4-D + 0–1 mg L^{-1} kinetin, dark) for callogenesis; and 1 mg L^{-1} IBA + 1 mg L^{-1} kinetin for embryogenesis. This study demonstrates that in vitro culture conditions can be fine-tuned to selectively enhance the production of specific secondary metabolites. For instance, dark conditions combined with a high concentration of 2,4-D are ideal for phenolic extraction, compounds known for their antioxidant, anti-inflammatory, and UV-protective properties. Conversely, light exposure combined with kinetin-rich media promotes flavonoid and anthocyanin accumulation, metabolites with applications in natural dyes, nutraceuticals, and cosmeceuticals.

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Author Contributions

Designed the study, EC; Performed the experiments, VAQ; Data collection and wrote the manuscript, EC, YPH, and VGO; analysed the data, VAQ; interpretation interpreted the research results, MM and YPH; supervised the experiments, EC, YPH, VGO, and MM. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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