An efficient in vitro regeneration system via callus from hypocotyl and root of *Iris laevigata*

Fengyi Li

College of Landscape Architecture, Northeast Forestry University <https://orcid.org/0000-0002-2717-4559>

Lijuan Fan

College of Landscape Architecture, Northeast Forestry University

Haijing Fu

College of Landscape Architecture, Northeast Forestry University

Yujia Liu

College of Landscape Architecture, Northeast Forestry University

Ling Wang (✉ wanglinghlj@126.com)

<https://orcid.org/0000-0001-8917-7889>

Research

Keywords: *Iris laevigata*, Plant regeneration, Callus, Plant growth regulator, Bacterial endophytes

Posted Date: July 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-46031/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Iris laevigata is an ornamental plant with strong cold resistance. However, its low reproductive capacity limits its landscape applications. The *I. laevigata* wild genetic resources also need to be protected. In order to develop an effective regeneration system, the optimum agar concentration for the induction medium was determined. Two explants (hypocotyl and root) were then cultured on medium containing different concentrations of plant growth regulator (PGR). In addition, three antibiotics were evaluated for controlling endophyte contamination.

Results

The highest induction rate (75.00%) was obtained from hypocotyl explants on Murashige and Skoog salt mixture (MS) medium containing 6-benzylaminopurine (6-BA) 0.5 mg L⁻¹ + 2,4-Dichlorophenoxyacetic acid (2,4-D) 1.0 mg L⁻¹ + 1-naphthylacetic acid (NAA) 0.4 mg L⁻¹. The medium containing 6-BA 0.5 mg L⁻¹ + 2,4-D 0.5 mg L⁻¹ + NAA 0.2 mg L⁻¹ achieved the greatest multiplication rate (73.33%). Media containing indole-3-butyric acid (IBA) 0.5 mg L⁻¹ + 6-BA 1.5 mg L⁻¹ + NAA 1.0 mg L⁻¹ achieved the highest differentiation rate (39.72%) for hypocotyl induced calli. Medium containing 6-BA 2.0 mg L⁻¹ + NAA 0.4 mg L⁻¹ + kinetin (KT) 1.0 mg L⁻¹ resulted in the highest differentiation rate (49.52%) for root induced calli. One hundred mg L⁻¹ penicillin G resulted in the optimal rate for reducing endophyte contamination.

Conclusions

The research determined the optimum PGR concentrations for inducing and multiplying *I. laevigata* calli from hypocotyl and root explants and a satisfactory means for control endophyte contamination. This research will result in the efficient and reliable reproduction of *I. laevigata* for landscape applications, genetic development of new *Iris* varieties, and preservation of the wild genetic material.

Background

I. laevigata is a perennial herbaceous flowering plant of *Iridaceae*, which has attracted increasing attention in recent years. In the wild, it is primarily found along rivers, in marshes and wetlands in China, Japan, South Korea, and Russia [1, 2]. Due to its gorgeous blue flowers, which are larger than many *Iridaceae* species, it is regarded as an aquatic plant with high ornamental value [3]. It has a long history of cultivation, and a common ornamental plant in northeastern China, and many renowned botanical gardens around the world [4]. Because it has outstanding adaptability, it can be used in both land and water landscaping. In addition to its landscape applications, *I. laevigata* is also the parent of many popular horticultural varieties, and, therefore, considered a significant genetic resource for iris breeding. Because of its native ecological origins, it has a strong tolerance for cold and waterlogged conditions.

These physiological characteristics make it a valuable germplasm resource for developing new ornamental iris varieties, which will be adapted to waterlogged and cold conditions in landscaping areas [5–8].

Under natural conditions, *Iris* species reproduce by two methods: asexually by splitting bulbs or rhizomes, and sexually by seeds. However, neither of these two methods can achieve large-scale and efficient reproduction. Ramets not only have a low reproduction coefficient, but also cannot guarantee uniform seedling quality. In addition, researchers [9, 10] continue to encounter consistent challenges to reproduce *I. laevigata* plants via seed germination. Furthermore, the increasing loss and damage of *I. laevigata* habitats pose a critical challenge to the survival of native wild populations. It has been defined as one of the second grade endangered species in South Korea [11].

Micropropagation is a method to regenerate new plants that can be used rapidly for ornamental horticulture applications. It is also a viable strategy for increasing the number of individuals ex-situ in order to save endangered species [12]. Moreover, the establishment of a callus regeneration system is also one of the basic conditions for plant genetic transformation and further development of plant genetic resources. Reliable callus regeneration systems have been developed for several iris species with high horticultural value or in danger of extinction [13–17], however, there is not a reliable callus regeneration system for *I. laevigata*. Therefore, we are committed to developing a callus regeneration system for *I. laevigata* that will help promote its horticultural applications and natural resource protection.

Endophytes often affect the growth of explants and calli in plant tissue cultures and their impact varies depending on the plant species [18–20]. At the beginning of the experiment, we discovered that *I. laevigata* calli were often contaminated by transparent and sticky endophytes in the multiplication medium. If the endophytes were left untreated, the calli would gradually become mushy or turn brown and die, which obviously seriously reduced the callus regeneration efficiency.

In this research, two explants, hypocotyl and root, were used to develop a suitable medium for callus induction, proliferation, and differentiation. The survival rate of regenerated plants was used to evaluate the experimental treatments. In addition, three broad-spectrum antibiotics were tested to develop an effective endophyte inhibition method for *I. laevigata* micropropagation. As a result of the research, an efficient and reliable callus regeneration system for *I. laevigata* was successfully developed.

Results

Effect of agar content in medium on hypocotyl callus induction

Hypocotyls were harvested from 20 d old sterile *I. laevigata* seedlings and used as explants to induce calli in media with different agar concentrations. The callus induction rate, endogenous bacterial contamination rate, and callus appearance were recorded after 30 d (Table 1). The medium callus

induction rate with 6 and 7 g L⁻¹ agar was significantly greater than that of other treatments (4 and 10 g L⁻¹). The calli induced on the 4 g L⁻¹ agar medium were severely contaminated with endophytes (66.67 ± 8.82%), while the contamination rate for other medium formulations were significantly less and not significantly different from each other.

The agar concentration also affected the appearance and moisture levels of the calli (Table 1). As the agar content increased from 4 to 10 g L⁻¹, the calli transformed from soggy/mushy to extremely dry. When the agar content was low (4 g L⁻¹), the calli were soggy and became mushy. At 6 g L⁻¹, the calli contained less moisture, but remained soggy. The calli appeared ideal, dry and granular, at 7 g L⁻¹, which also produced a high, 56.67%, induction rate and lower (20%) contamination rate. In contrast, the 10 g L⁻¹ agar produced calli that were extremely dry with a low induction rate (23.33%). As a result, it was determined that adding 7 g L⁻¹ agar to the medium produced the most suitable callus formations for *I. laevigata* hypocotyl explants.

Table 1
Effect of different agar content on *I. laevigata* hypocotyl callus induction

Agar content (g L ⁻¹)	Induction rate (%)	Contamination rate (%)	Callus appearance
4	33.33 ± 3.33 b	66.67 ± 8.82 a	severely contaminated and mushy
6	53.33 ± 3.33 a	23.33 ± 3.33 b	soggy
7	56.67 ± 6.67 a	20.00 ± 5.77 b	dry and granular
10	23.33 ± 8.82 b	16.67 ± 6.67 b	extremely dry

Each value represents the mean ± standard error (SE) of three independent experiments, each of which includes 10 hypocotyl explants. Different lowercase letters in the same column indicate significant differences at *P* ≤ 0.05 as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Effects of different PGRs concentrations on *I. laevigata* hypocotyl callus induction

Sterile *I. laevigata* seedling hypocotyls (20 d old) were used as explants and placed flat on medium with different hormone concentrations to induce callus development. The callus induction rate, endogenous bacterial contamination rate, and callus appearance were recorded after 45 d (Table 2; Fig. 1A). Calli were formed at the incision at the top and bottom of the hypocotyl as well as at the inevitable incisions on the hypocotyl during the experimental operation (Fig. 1B). Treatment 2's callus induction rate was 75%, and the induced calli were in good condition (Table 2). Therefore, the optimal medium for *I. laevigata* hypocotyl callus induction was 6-BA 0.5 mg L⁻¹ + 2,4-D 1.0 mg L⁻¹ + NAA 0.4 mg L⁻¹ + MS + sucrose 30 g L⁻¹ + agar 7 g L⁻¹.

Table 2
Effects of different PGRs concentrations on hypocotyl induced calli and range analysis

Treatment	Orthogonal array			PGRs (mg L ⁻¹)			Induction rate (%)	Contamination rate (%)	Callus appearance
	A	B	C	6-BA	2,4-D	NAA			
1	1	1	1	0.5	0.5	0.2	55.00 ± 2.24 bc	0	yellow, dry, and granular
2	1	2	2	0.5	1.0	0.4	75.00 ± 2.24 a	0	yellow, dry, and granular
3	1	3	3	0.5	1.5	0.6	43.33 ± 3.33 c	0	yellow, dry, and granular
4	2	1	2	1.0	0.5	0.4	48.33 ± 4.01 c	0	yellow, dry, and granular
5	2	2	3	1.0	1.0	0.6	61.67 ± 1.67 b	0	yellow, dry, and lumpy
6	2	3	1	1.0	1.5	0.2	51.67 ± 1.67 bc	0	yellow, dry, and lumpy
7	3	1	3	2.0	0.5	0.6	23.33 ± 6.67 d	0	yellow, dry, and granular
8	3	2	1	2.0	1.0	0.2	28.33 ± 5.43 d	0	yellow, dry, and granular
9	3	3	2	2.0	1.5	0.4	21.67 ± 6.54 d	0	yellow, dry, and granular
K1	-	-	-	57.78	42.22	45	-	-	-
K2	-	-	-	53.89	55.00	48.33	-	-	-
K3	-	-	-	24.44	38.89	42.78	-	-	-
R	-	-	-	33.34	16.11	5.55	-	-	-

Each value represents the mean ± SE of three independent experiments, each of which includes 20 hypocotyl explants. Different lowercase letters in the same column indicate significant differences at $P \leq 0.05$ as determined by one-way analysis of variance (ANOVA) with Duncan's post-test. Kx means sum of induction rate of each factor at x level; R means measures of variation, $R = K_{\max} - K_{\min}$.

As the 6-BA concentration increased from 0.5 mg L⁻¹ to 2.0 mg L⁻¹, the mean induction rate decreased (Table 2; Fig. 2). As the 2,4-D concentration increased from 0.5 mg L⁻¹ to 1.5 mg L⁻¹ the mean value of the induction rate first significantly increased and then decreased. The impact of NAA concentration followed the same general trend as 2,4-D, which initially increased and then decreased as NAA concentration increased, although the differences were not significant (Fig. 2). Therefore, it was

determined that the most suitable phytohormone combination was 6-BA 0.5 mg L⁻¹ + 2,4-D 1.0 mg L⁻¹ + NAA 0.4 mg L⁻¹, which produced a 75% callus induction rate (Table 2). Furthermore, according to the range analysis (Table 2), the influence of the PGRs on hypocotyl callus induction are ranked as 6-BA > 2,4-D > NAA. The analysis of variance (Table 3) indicated that 6-BAP was the most significant factor involved in the hypocotyl induction rate ($P < 0.01$), while 2,4-D affected the induction rate to a lesser extent ($P < 0.05$), and NAA had relatively little influence ($P > 0.05$).

Table 3
Variance analysis of PGRs influence on the *I. laevigata* hypocotyl callus induction rate

Factor	Sum of squares	df	Mean square	F	P-value
6-BA	11959.259	2	5979.630	35.529	0.000
2,4-D	2603.704	2	1301.852	3.701	0.032
NAA	281.481	2	140.741	0.354	0.703

Effects of different PGRs concentrations on *I. laevigata* seedling root callus induction.

To determine the impact of 3 phytohormones on *I. laevigata* root callus induction, 2 types of *I. laevigata* root explants were investigated. One set of root explants had no fibrous roots, while the second root explants had the fibrous roots removed. Root explant segments without fibrous roots did not produce any calli, independent of the phytohormone concentration in the growth medium. Therefore, this manuscript will only discuss the callus induction rate from root explants which the fibrous roots were originally attached but removed (Fig. 3; Table 4).

Explant root segments which originally had fibrous roots were collected from sterile *I. laevigata* seedlings (50 d old) and placed in media containing 3 different phytohormones (6-BA, 2,4-D, and NAA). Table 4 shows the callus induction rate and callus appearances resulting from the various PGR concentrations. When the 6-BA and NAA concentrations were held constant, the root callus induction rate increased as the 2,4-D concentration increased, resulting in a single peak curve (Table 4; Fig. 3). A single peak curve was also produced when the 6-BA and 2,4-D concentrations were held constant and the NAA concentration increased (Table 4; Fig. 3). The greatest callus induction rates for root explants were produced when the media contained 2,4-D at 0.5 mg L⁻¹ and NAA at 0.4 mg L⁻¹ with either 0.5 or 2.0 mg L⁻¹ 6-BA (Table 4; Fig. 3). When the media did not contain 6-BA (treatment 1, 0 mg L⁻¹) there was no (0%) callus induction for the root explants (Table 4). When either 2,4-D or NAA were absent from the media (treatment 5 and 8), the callus induction rates were very low, 6.67% and 3.33%, respectively. As a result, it is clear that although 6-BA had the most significant impact on root callus induction rates, the presence of low rates of 2,4-D (0.5 mg L⁻¹) and NAA (0.4 mg L⁻¹) were essential for maximizing callus induction (Table 4). Therefore, to produce maximum callus induction rates with *I. laevigata* root explants, the medium must contain the 3 PGRs (6-BA, 2,4-D, and NAA) rather than just 2 PGRs. Although callus induction rates for treatment 2 (6-BA 0.5 mg L⁻¹ + 2,4-D 0.5 mg L⁻¹ + NAA 0.4 mg L⁻¹) and treatment 3 (6-BA 2.0 mg L⁻¹ + 2,4-D 0.5 mg L⁻¹ + NAA 0.4 mg L⁻¹) were not significantly different, 73.33% and 66.67%, respectively,

treatment 2 required less 6-BA and produced numerically greater callus inductions at 45 d (Table 4). As a result, the author's recommend the following medium formulation for *I. laevigata* root callus induction: 6-BA 0.5 mg L⁻¹ + 2,4-D 0.5 mg L⁻¹ + NAA 0.4 mg L⁻¹ + MS + sucrose 30 g L⁻¹ + agar 7 g L⁻¹.

Table 4
Effects of different PGRs concentrations on *I. laevigata* root induced callus formation

Treatment	6-BA (mg L ⁻¹)	2,4-D (mg L ⁻¹)	NAA (mg L ⁻¹)	Induction rate (%)	Callus appearance
1	0	0.5	0.4	0.00 ± 0.00 d	No callus
2	0.5	0.5	0.4	73.33 ± 9.89 a	yellow, small
3	2.0	0.5	0.4	66.67 ± 12.29 a	yellow, small
4	3.0	0.5	0.4	26.67 ± 4.22 bc	yellow, small
5	0.5	0	0.4	6.67 ± 4.22 d	yellow, small
6	0.5	1.0	0.4	36.67 ± 3.33 b	yellow, small
7	0.5	2.0	0.4	16.67 ± 3.33 cd	yellow, small
8	0.5	0.5	0	3.33 ± 3.33 d	yellow, small
9	0.5	0.5	1.0	30.00 ± 4.47 bc	yellow, small
10	0.5	0.5	2.0	13.33 ± 4.21 cd	yellow, small

Each value represents the mean ± SE of three independent experiments, each of which included 20 explants. Different lowercase letters in the same column indicated the significant difference at $P \leq 0.05$ as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Effects of different PGRs and concentrations on root callus multiplication

The callus was inserted into the multiplication medium of different PGRs concentrations for 45 d (Table 5; Fig. 4).

Interestingly, there were no significant differences in the endophyte contamination rates among the experimental groups with different PGR content. All PGR combinations resulted in multiplication rates equal to or greater than 21.67%, with the greatest numerical value of 73.33%. The multiplication rates were inversely proportional to the NAA concentrations. As 6-BA and 2,4-D were held constant at 0.5 mg L⁻¹ and the NAA concentration increased from 0.2 to 0.6 mg L⁻¹, the multiplication rate decreased from 73.33–28.33%. At the same NAA concentration (0.4 mg L⁻¹), the multiplication rate was greater at the lowest (0.5 mg L⁻¹) 6-BA and 2,4-D concentrations. The two highest multiplication rates were at the two lowest PGR concentrations, treatment 1 and 2. As a result, it was decided that treatment 1 (6-BA 0.5 mg

L^{-1} + 2,4-D 0.5 mg L^{-1} + NAA 0.2 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 8 g L^{-1}), with a multiplication rate of 73.33%, was the most suitable medium formula for root callus multiplication.

Table 5
Effects of different PGRs combinations on root callus multiplication

Treatment	6-BA (mg L^{-1})	2,4-D (mg L^{-1})	NAA (mg L^{-1})	Multiplication rate (%)	Contamination rate (%)	Callus appearance
1	0.5	0.5	0.2	73.33 ± 2.11 a	33.33 ± 14.53 a	yellow, dry, and granular
2	0.5	0.5	0.4	61.67 ± 1.67 b	26.67 ± 3.33 a	yellow, dry, and granular
3	0.5	0.5	0.6	28.33 ± 3.07 de	28.33 ± 4.77 a	yellow, dry, and granular
4	1.0	1.0	0.4	21.67 ± 1.67 e	26.67 ± 4.22 a	yellow, dry, and granular
5	2.0	0.5	0.4	38.33 ± 3.07 cd	28.33 ± 3.07 a	green spots on the surface, showing a trend of differentiation
6	1.5	0.5	0.4	46.67 ± 6.67 c	31.67 ± 4.77 a	yellow, dry, and granular

Each value represents the mean $\pm$ SE of three independent experiments, each of which includes 20 root explants. Different lowercase letters in the same column indicated the significant difference at $P \leq 0.05$ as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Effects of different PGRs and concentrations on hypocotyl and root callus differentiation

Three media formulations were evaluated to determine their impact on hypocotyl callus differentiation (Table 6). The greatest differentiation rate (39.72%) was achieved after 60 d with a medium containing IBA 0.5 mg L^{-1} + 6-BA 1.5 mg L^{-1} + NAA 1.0 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 8 g L^{-1} (Table 6). These results confirm that IBA is significantly involved in hypocotyl callus differentiation. No adventitious shoots were produced without adding IBA to the media and the resulting shoots were green and strong when 0.5 mg L^{-1} IBA was added to the media.

Table 6
Effects of different PGR combinations on differentiation of hypocotyl induced calli.

Treatment	IBA (mg L ⁻¹)	6-BA (mg L ⁻¹)	NAA (mg L ⁻¹)	Differentiation rate (%)	Adventitious shoot appearance
1	0	1.0	0.4	00.00 ± 0.00 c	Callus Browning
2	0.5	1.0	0.4	16.67 ± 8.33 b	green, strong
3	0.5	1.5	1.0	39.72 ± 15.28 a	green, strong

Each value represents the mean ± SE of three independent experiments, each of which includes 20 hypocotyl explants. Different lowercase letters in the same column indicated the significant difference at $P \leq 0.05$ as determined by one-way analysis of variance (ANOVA) with Duncan's post-test.

Root induced calli were placed into the differentiation media, which included various concentrations of 6-BA, NAA and KT (kinetin), and were cultured for 60 d (Table 7). The maximum differentiation rate for root induced calli was 49.52%, treatment 7, 6-BA 2.0 mg L⁻¹ + NAA 0.4 mg L⁻¹ + KT 1.0 mg L⁻¹ + MS + sucrose 30 g L⁻¹ + agar 8 g L⁻¹. The calli produced were green with strong adventitious shoots.

Table 7
Effects of different PGRs combinations on differentiation of root induced calli and range analysis

Treatment	Orthogonal array			PGRs (mg L ⁻¹)			Differentiation rate (%)	Adventitious shoot appearance
	A	B	C	6-BA	NAA	KT		
1	1	1	1	1.0	0.4	0	7.50 ± 4.79 c	greenish yellow, thin
2	1	2	2	1.0	0.6	0.5	15.72 ± 6.12 bc	greenish yellow, thin
3	1	3	3	1.0	0.8	1.0	23.33 ± 3.33 bc	deep green, strong
4	2	1	2	1.5	0.4	0.5	16.67 ± 8.03 bc	green, strong
5	2	2	3	1.5	0.6	1.0	26.67 ± 4.22 bc	green, strong
6	2	3	1	1.5	0.8	0	13.33 ± 4.22 bc	green, strong
7	3	1	3	2.0	0.4	1.0	49.52 ± 11.13 a	deep green, strong
8	3	2	1	2.0	0.6	0	26.67 ± 4.22 bc	deep green, strong
9	3	3	2	2.0	0.8	0.5	30.00 ± 4.47 b	green, thin
K1	-	-	-	15.52	24.56	15.83	-	-
K2	-	-	-	18.89	23.02	20.80	-	-
K3	-	-	-	35.40	22.22	33.17	-	-
R	-	-	-	19.88	2.34	17.34	-	-

Each value represents the mean ± SE of three independent experiments, each of which includes 20 root explants. Different lowercase letters in the same column indicated the significant difference at $P \leq 0.05$ as determined by one-way analysis of variance (ANOVA) with Duncan's post-test. 3 Kx means sum of induction rate of each factor at x level; 4 R means measures of variation, $R = K_{\max} - K_{\min}$.

Within a certain concentration range, the induction rate was directly proportional to the 6-BA and KT concentrations, and inversely proportional to the concentration of NAA (Fig. 5). According to the range analysis (Table 7), the influence of each factor was ranked as 6-BA > KT > NAA. The analysis of variance indicated that both 6-BA and KT had a significant impact on the differentiation rate ($P < 0.01$), while NAA had relatively little influence ($P > 0.05$) (Table 8). Therefore, future research should investigate the use only the two PGRs, 6-BA and KT, for *I. laevigata* root differentiation medium.

Table 8
Variance analysis of PGRs influence on the differentiation rate of root induced calli

Factor	Sum of squares	df	Mean square	F	P-value
6-BA	4074.713	2	2037.357	7.900	0.001
NAA	51.030	2	25.515	0.076	0.927
KT	2871.596	2	1435.798	5.101	0.010

Rooting and transplanting of adventitious shoots

The adventitious shoots were multiplied, then pruned to a suitable height, and placed in the rooting culture medium. After 30 d, the regenerated seedlings were cultured in water and then transplanted to potting soil. The potted seedlings were then placed in a greenhouse for 30 d and maintained a 90% survival rate.

The impact of three antibiotics on the control of endophytes in calli

During the experiment, it was found that the endophytes severely inhibited the induction and multiplication of calli. Therefore, we investigated the impact of adding different combinations of penicillin G (50, 100, 200, 300 mg L⁻¹), carbenicillin disodium (50, 100, 300, 500 mg L⁻¹) and cefotaxime sodium (50, 100, 300, 500 mg L⁻¹) to media to control endophytes. Each antibiotic had a positive impact on endophytic bacteria control (Fig. 6). The penicillin G bacteriostatic rate was significantly greater than the other two antibiotics at all concentration levels (Fig. 6). The inhibition rate increased gradually as the penicillin G concentration increased and reached the greatest inhibition (54.29%) at the 300 mg L⁻¹ concentration (Fig. 6A).

Besides controlling the endophytes, antibiotics also have the potential to negatively impact plant materials and cause callus browning and death. Each of the three antibiotics resulted in a varying degrees of browning (Fig. 6B). Overall, penicillin G produced the least browning compared to other two antibiotics, carbenicillin disodium and cefotaxime sodium. The lowest two penicillin G concentrations, 50 and 100 mg L⁻¹, produced the lowest browning rates. As the penicillin G concentration increased from 100 to 300 mg L⁻¹, the browning rate significantly increased (Fig. 6B). Therefore, penicillin G was selected as the superior antibiotic to both control endophytes and limit callus browning (Fig. 6A; 6B). The addition of penicillin G at 100 mg L⁻¹ provided the best balance between inhibiting endophytes and limiting browning (Fig. 6A; 6B; 6C).

Discussion

In tissue culture, the concentration of gelling agent has an important effect on the induction rate and callus growth [21–23]. Differences in gel strength, mineral content, and inhibition compounds can also influence callus growth [24]. This research evaluated the effect of agar content on the growth of explants and calli. As the agar content increased, the media strength and the degree of drying increased. In addition, as agar content increased, the induction rate increased and the callus granulation became more obvious. Interestingly, in the process, the contamination rate decreased. The greatest callus induction rate was achieved (56.67%) when the agar content reached 7 g L^{-1} . However, when the agar concentration increased to 10 g L^{-1} , the medium became too dry, which resulted in a decrease in the callus induction rate. Other researchers [21, 25] have shown that the optimal agar concentration will change according to the species and genotype. In our research we determined that the optimum agar concentration for *I. laevigata* callus induction was 7 g L^{-1} . Furthermore, different kinds of gelling agents, such as gellan gum [26] and phytigel [23], can also result in differences in plant regeneration.

The source of explants has a decisive influence on the ability and efficiency for in vitro regeneration. The capacity of callus induction of different explants is very diverse, depending on their genotype and growth status [27]. Stem tips, leaf bases, floral organs, rhizomes, and roots are often used for callus regeneration research with *Iris* species [14–16, 28–31]. Two explants, hypocotyls and roots, were investigated in this research, and there was no significant difference in callus induction rate between the two types of explants, 75% and 73.33%, respectively. However, the differentiation rates for the hypocotyl and root explants were 39.72% and 49.52%, respectively. The lower differentiation rate for hypocotyl explants (39.72%) compared to root explants (49.52%) needs further experimentation to increase the hypocotyl differentiation rate. Future research could investigate additional PGR combinations and concentrations. Additionally, Li et al. [32] reported that *Brassica oleracea* var. *capitata* calli were induced with fibrous root explants (15, 20, and 25 days old). Initially, the calli appeared at the incision sites at both ends of the root segment, and then the calli were induced over the entire explant. The fibrous root explants produced the greatest induction rate (96.00%) at 20 d [32]. In our research, the thicker adventitious roots were selected as explants on which the thinner fibrous roots were excised. Unlike Li et al. [32] research, the *I. laevigata* root induced calli appeared at only one root end (Fig. 7A), but did produce calli at the fibrous root excise locations along the root (Fig. 7B). It is speculated that the roots used for root explants were too old (30 d) after aseptic seedling process, therefore, resulting in the decline callus induction. However, the fibrous root explants were more tender and readily resulted in calli.

The types, concentrations, and combinations of plant growth regulators play an important role in plant regeneration [33, 34]. Moreover, even for the same plant species, different explants respond differently depending on the type and level of PRGs [35]. Analysis of variance for the hypocotyl induced callus results indicated that 6-BA and 2,4-D were relatively important factors affecting the induction rate, while NAA had little effect. However, when root segments were used to induce calli, the induction rate was very low if only two PGRs (6-BA, 2,4-D or NAA) were used. The combination of the three PRGs was more conducive to callus induction than any combination of two PRGs. In the callus differentiation culture, NAA had little impact on the differentiation of root induced calli among the three PRGs (6-BA, KT, and

NAA). Additional research should be conducted with only 6-BA and KT, and other PGR combinations and concentrations to increase the differentiation rate for root induced calli.

Also, the hypocotyl induced calli did not differentiate when 6-BA 1.0 mg L^{-1} and NAA 0.4 mg L^{-1} were added to the medium, but the differentiation rate increased when IBA 0.5 mg L^{-1} was added. Obviously, IBA played a key role in the differentiation of adventitious shoots. Similar results have been shown concerning auxins being crucial for adventitious shoot differentiation [36, 37]. This is because auxins can rapidly regulate cytokinin synthesis [36, 37]. In conclusion, the balance between culture medium PGRs and PGRs in explants and calli are consequential.

Endophytes that would not cause a disease when living in plant tissue [38], can often lead to explant browning and necrosis in calli in plant regeneration systems [19, 20]. It is well established that adding antibiotics to the culture medium is an effective method to eliminate endophyte contamination in plant tissue cultures [20, 39, 40]. In this research, we studied the impact of three antibiotics (penicillin G, carbenicillin disodium, and cefotaxime sodium) on endophyte contamination. We determined that adding penicillin G 100 mg L^{-1} to the culture medium was the best strategy to inhibit endophyte contamination. Although, we did not identify the endophytes involved in our research, researchers often use targeted antimicrobial agents to control known endophyte species [39, 41]. For example, Han et al. [42] determined that *Staphylococcus epidermidis* was responsible for the contamination of *I. germanica* in micropropagation. Future research should investigate which endophyte species are involved in *I. laevigata* contamination to better target the control of the endophytes.

Conclusions

This is the first report on the in vitro regeneration system of calli from *I. laevigata*. It was determined that there was no significant difference in callus induction rates between the two explants (hypocotyl and root). The greatest hypocotyl callus induction rate (75%) was achieved with 6-BA 0.5 mg L^{-1} + 2,4-D 1.0 mg L^{-1} + NAA 0.4 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 7 g L^{-1} . The greatest root callus induction rate (73.33%) was achieved with 6-BA 0.5 mg L^{-1} + 2,4-D 0.5 mg L^{-1} + NAA 0.4 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 7 g L^{-1} . The optimum medium for callus multiplication was MS + 6-BA 0.5 mg L^{-1} + 2,4-D 0.5 mg L^{-1} + NAA 0.2 mg L^{-1} , and the proliferation rate was 73.33%. The greatest hypocotyl differentiation rate (39.72%) was achieved with IBA 0.5 mg L^{-1} + 6-BA 1.5 mg L^{-1} + NAA 1.0 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 8 g L^{-1} . And the greatest root differentiation rate (49.52%) was achieved with 6-BA 2.0 mg L^{-1} + NAA 0.4 mg L^{-1} + KT 1.0 mg L^{-1} + MS + sucrose 30 g L^{-1} + agar 8 g L^{-1} . The most effective antimicrobial agent for *I. laevigata* callus multiplication was penicillin G 100 mg L^{-1} . These results will be advantageous for the micropropagation and commercial horticultural application of *I. laevigata* and other *Iris* species.

Methods

Experimental method

In this research, we established an *I. laevigata* callus regeneration system using hypocotyls and root segments as explants. The optimal medium agar content for callus induction was determined by the assessing the callus induction rate, endophyte contamination rate, and hypocotyl callus growth. In addition, the research identified the formula for adding phytohormones to the medium to maximize callus induction, multiplication, and differentiation for two *I. laevigata* explant seedling sources (hypocotyls and roots). The differentiated adventitious shoots were then rooted and transplanted. Standard MS medium was used in the experiment with the addition of sucrose at 30 g L^{-1} . All experiments were conducted in a tissue culture chamber at $25 \pm 1 \text{ }^{\circ}\text{C}$ with a light intensity of $25 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ for 14 h d^{-1} .

Explant sources

Seeds of *I. laevigata* were collected in September 2018 from Tahe, Heilongjiang province, China, and then stored in low-temperature sand in the Northeast Forestry University experimental nursery from October to December 2018. After December 2018, the seeds were kept in a $4 \text{ }^{\circ}\text{C}$ refrigerator. The seeds were sterilized with 75% alcohol for 10 s and 2% NaOCl for 25 min, followed by washing with sterile deionized water and then inoculated into the culture dish with wet filter paper. They were then cultured for 20 d until sprouting 2–3 leaves and roots. The leaves and roots of the sterile seedlings (20 d old) were removed, leaving a hypocotyl about 1 cm long to be used as explants. In addition, sterile seedlings (20 d old) were cultured in rooting medium for 30 d, and then the roots were cut off and the fibrous roots were removed. These roots were then cut into 1 cm long segments and used as explants. All the operations were carried out in sterile conditions.

Medium preparation

Standard MS medium was used in all the experiments with sucrose added at 30 g L^{-1} . The various PGRs at assigned concentrations were added to media, along with antibiotics according the experimental design. The media were then adjusted to pH 5.9 and sterilized at $121 \text{ }^{\circ}\text{C}$ by autoclaving after adding different agar concentrations. All Petri dishes used in the experiments were sterilized at $121 \text{ }^{\circ}\text{C}$ by autoclaving. Thirty ml of each medium was poured into separate sterilized Petri dishes, allowed to cool to coagulate, and sealed with Parafilm in a sterile environment. The Petri dishes were then stored for 3 d and observed to confirm their sterility prior to being used.

Determination of agar content in callus induction medium

Hypocotyl explants were inserted into media with 4, 6, 7, and 10 g L^{-1} of agar to induce callus formation. The induction rate, endophyte contamination rate, and callus appearance were recorded after 30 d (Table 1). Each treatment included 10 explants and three replications.

Callus induction from two explants

The explants, hypocotyls and root segments, were placed into the medium horizontally and assure that the explants were in full contact with the medium. The induction rate and callus appearance were

evaluated after 45 d (Table 2; Table 4). Each treatment included 20 explants and three replications.

Callus multiplication

Healthy calli of almost uniform size were selected and inoculated on the multiplication media with the different experimental formulations (Table 5). The optimal multiplication medium was determined by comparing the multiplication rate, the contamination rate, and the callus appearance after 45 d. Each treatment included 20 explants and three replications.

Callus differentiation

Healthy calli of almost uniform size were selected and inoculated on the differentiation medium with different experimental formulas (Table 6; Table 7). After being cultured for 60 d, the differentiation rate and adventitious shoot appearance were recorded. Each treatment included 20 explants and three replications.

Multiplication, rooting, and transplanting of adventitious shoots

The adventitious shoots were transferred to MS medium with IBA 0.5 mg L⁻¹ + 6-BA 1.5 mg L⁻¹ + NAA 1.0 mg L⁻¹ + sucrose 30 g L⁻¹ + agar 9 g L⁻¹ for further subculture. Subsequently, the clumps were separated into 4 adventitious shoots and their leaves were clipped to 2 cm. The treated shoots were inoculated on the MS medium containing NAA 0.2 mg L⁻¹ for rooting. After 30 d of rooting, the culture bottle caps were removed and added to sterile deionized water to acclimate the in vitro regenerated plantlets. Three days later, the plantlets were removed from the bottles and washed carefully to remove any residual medium from the roots. The plantlets were grown hydroponically for 20 d after cleaning the roots and leaves. The seedlings were then transplanted into pots filled with orchard soil and vermiculite (3:1). The survival rate of transplanted plants was calculated after 30 d.

Elimination of Endophytes by antibiotics

The calli with endophytic bacteria were placed into multiplication media, which included different combinations of penicillin G (50, 100, 200, 300 mg/L), carbenicillin disodium (50, 100, 300, 500 mg/L) and cefotaxime sodium (50, 100, 300, 500 mg/L). The inhibition and browning rates of calli were monitored to determine the most suitable antimicrobial agents for *I. laevitaga* calli. Each treatment included 20 explants and three replications.

Statistical analysis

Induction rate (%) = the number of induced explants/the number of total inserted explants × 100%

Contamination rate (%) = the number of explants contaminated by endophytes/the number of total inserted explants × 100%

Multiplication rate (%) = the number of multiplied calli/the number of total inserted calli × 100%

Differentiation rate (%) = the number of differentiated calli/the number of total inserted calli × 100%

Browning rate (%) = the number of browning explants/the number of total inserted explants × 100%

Survival rate (%) = the number of plantlets survived after transplanting/the number of total transplanted plantlets × 100%

Each treatment was repeated three independent times. All data represents the mean value and standard error of three replicates. Data analyses were performed using SPSS 22.0 software. One-way analysis of variance (ANOVA) was used to determine the significance of the various effects studied. Duncan's Multiple Range Test was used for pair-wise comparison of the data.

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid; 6-BA: 6-benzylaminopurine; IBA: indole-3-butyric acid; KT: kinetin; MS: Murashige and Skoog salt mixture; NAA: 1-naphthylacetic acid; PGR: plant growth regulator; SE: standard error;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Natural Science Foundation of China (No.31670344) and Fundamental Research Funds for the Central Universities (No.) .

Authors' contributions

FYL, LJF, YJL, and LW designed the study. FYL, LJF, HJF, and YJL performed the experiments. FYL and YJL analyzed the results. FYL, YJL, and HJF wrote the manuscript, and LW provided advice. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the College of Landscape Architecture of Northeast Forestry University for providing support for the establishment of tissue culture conditions, and all laboratory members for their useful suggestions.

References

1. Sun MZ, Li MR, Shi FX, Li L, Liu Y, Li LF, et al. GENOMIC AND EST-DERIVED MICROSATELLITE MARKERS FOR IRIS LAEVIGATA (IRIDACEAE) AND OTHER CONGENERIC SPECIES. *Am J Bot.* 2012;99(7):E286-E8.
2. Qu W, Qiao Y, Wang L, Tang Z. Responses of Morphology, Physiology and Reproduction of *Iris laevigata* to Change of Water Level. *Wetland Science.* 2018;16(1):79–84.
3. 孙明志, 李梦然, 李林, 刘颖, 李丽芳, et al. Morphological Characteristics and Genetic Relationship by RAPD Marker in *Iris* spp. *Journal of Plant Biotechnology.* 2004;31(1):19–23.
4. Wang LY, Wang HX, He CG, Sheng LX, Tang ZH. An irreversible division of labor through a sexually dependent system in the clonal plant *Iris laevigata* (Iridaceae). *Ecosphere.* 2017;8(3):11.
5. Inoue K, Tomita T, Yoshihara N, Yabuya T. Interspecific Hybrids between *Iris setosa* var. *setosa* and *I. laevigata* and Their Relationships to *I. setosa* var. *hondoensis* or *I. setosa* var. *nasuensis*. *Cytologia.* 2008;73(4):401–10.
6. Yabuya T, Nozaki T, Hanazaki A, Harada S, Tanaka H, Tomita T, et al. Origin of Japanese Endemic Species *Iris setosa* var. *nasuensis* and *I. setosa* var. *hondoensis*. *Cytologia.* 2013;78(4):449–59.
7. Yabuya T. Chromosome associations and crossability with *Iris ensata* Thunb. in induced amphidiploids of *I. laevigata* Fisch. × *I. ensata*. *Euphytica.* 1991;55(1):85–90.
8. Yabuya T. Chromosome association and fertility in hybrids of *Iris laevigata* Fisch. × *I. ensata* Thunb. *Euphytica.* 1984;33(2):369–76.
9. Diao X, Gao Y. Study on Dormancy and Germination of four *Iris* Species Seeds. *Seed.* 2006;25(4):41–4.
10. Lu M, Du Y, Bi X. Study on Seed Dormancy and Germination Characteristics with Five Species of Wild Apogons *Iris*. *Acta Botanica Boreali-Occidentalia Sinica.* 2017;37(9):1823–30.
11. 孙明志, 李梦然, 李林, 刘颖, 李丽芳, et al. Effects of water levels and soil nutrients on the growth of *Iris laevigata* seedlings. *Journal of Ecology Environment.* 2018;42(2):43–9.

12. Gonzalez-Benito ME, Martin C. In Vitro Preservation of Spanish Biodiversity. *In Vitro Cell Dev Biol-Plant*. 2011;47(1):46–54.
13. Hossain MM, Sharma M, da Silva JAT, Pathak P. Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Sci Hortic*. 2010;123(4):479–87.
14. Zhang Y, Yuan H, Gu C, Huang S. Preliminary selection of high frequency in vitro regeneration condition of *Iris foetidissima*. *Journal of Plant Resources Environment*. 2014;23(1):116–8.
15. Bae K-W, Yoo K-H, Lee H-B, Yoon E-S. Callus induction and plant regeneration of *Iris dichotoma* Pall. in endangered species. *Journal of Plant Biotechnology*. 2012;39(3):182–8.
16. Ge G, Hou J, Liu Z, Li J. Research on in Vitro Propagation of *Iris sibirica* L.Shoot Tip. *Journal of Henan Agricultral Sciences*. 2013;42(8):105–8.
17. Lin M, Kuo X, Mao-Lin Z, Guo-Fang Z. Technological System of Tissue Culture and Rapid Propagation of *Iris lactea* Pall. var. *chinensis* (Fisch.) Koidz. *Bulletin of Botanical Research*. 2009;29(2):193–7.
18. Regalado JJ, Berdion V, Vignale MV, Novas MV, Pitta-Alvarez SI, Iannone LJ. The presence of *Epichloe* sp in *Bromus auleticus* (Trin.) seeds enhances micropropagation and growth of micropropagated plantlets from these seeds. *Plant Cell Tissue Organ Cult*. 2018;135(2):279–86.
19. Miyazaki J, Tan BH, Errington SG, Kuo JJS. Bacterial endophyte in *Macropidia fuliginosa*: its localisation and eradication from in vitro cultured basal-stem callus. *Aust J Bot*. 2011;59(4):363–8.
20. Alagarsamy K, Shamala LF, Wei S. Influence of media supplements on inhibition of oxidative browning and bacterial endophytes of *Camellia sinensis* var. *sinensis*. *3 Biotech*. 2018;8(8):7.
21. Abdollahi MR, Najafi S, Sarikhani H, Moosavi SS. Induction and development of anther-derived gametic embryos in cucumber (*Cucumis sativus* L.) by optimizing the macronutrient and agar concentrations in culture medium. *Turk J Biol*. 2016;40(3):571–9.
22. Fu X, Fu Y, Liu G, He M, Shi Z. The Effect of the Agar Concentration in Different Medium on the Initiating Rate of the Anther Culture in Wheat. *Chin Agric Sci Bull*. 2014;30(15):46–9.
23. Duan YB, Su Y, Chao EK, Zhang GR, Zhao FL, Xue T, et al. Callus-mediated plant regeneration in *Isodon amethystoides* using young seedling leaves as starting materials. *Plant Cell Tissue Organ Cult*. 2019;136(2):247–53.
24. Scholten HJ, Pierik RLM. Agar as a gelling agent: chemical and physical analysis. *Plant cell reports*. 1998;17(3):230–5.
25. Malabadi RB, Van Staden J. Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. *Tree Physiol*. 2005;25(1):11–6.
26. Palanyandy SR, Gantait S, Sinniah UR. Effects of some gelling agents and their concentrations on conversion of oil palm polyembryoids into plantlets. *Journal genetic engineering biotechnology*. 2020;18(1):5.
27. Mostafa HHA, Wang H, Song J, Li X. Effects of genotypes and explants on garlic callus production and endogenous hormones. *Scientific reports*. 2020;10(1):4867.

28. Cerasela P, Simona L, Giancarla V, Marcel D, Alexandru L, Iuliana C, et al. Genetic stability of micropropagated *Iris germanica* L. varieties assessed by RAPD markers. *Rom Biotech Lett*. 2014;19(5):9778–84.
29. Chen C, Bi X, Lu M. Tissue Culture and Rapid Propagation of *Iris germanica* L. *Journal of Shenyang Agricultural University*. 2010;41(1):27–32.
30. Mengying LIU, Yang GA, ShiDa YU, Na XU, Changyang J. Study on Tissue Culture and Establishment of Asexual System of Chinese Iris. *Liaoning Agricultural Sciences*. 2007(6):4–6.
31. Jevremovic S, Radojevic L. Establishment of efficient regeneration protocol from leaf explants of *Iris pumila* shoots cultured in vitro. *Sci Hortic*. 2006;108(1):100–3.
32. Li N, Zhang E, Xu Z, Chen L, Jiang J, Cao L. Plant regeneration technology of *Brassica oleracea* var. capitata root. *Journal of Northwest A F University Natural Science Edition*. 2019;47(2):103–11.
33. Karakas FP, Turker AU. Improvement of shoot proliferation and comparison of secondary metabolites in shoot and callus cultures of *Phlomis armeniaca* by LC-ESI-MS/MS analysis. *In Vitro Cell Dev Biol-Plant*. 2016;52(6):608–18.
34. Wang L, Du Y, Rahman MM, Tang B, Fan LJ, Kilaru A. Establishment of an efficient in vitro propagation system for *Iris sanguinea*. *Sci Rep*. 2018;8:10.
35. Karakas FP. Efficient plant regeneration and callus induction from nodal and hypocotyl explants of goji berry (*Lycium barbarum* L.) and comparison of phenolic profiles in calli formed under different combinations of plant growth regulators. *Plant Physiol Biochem*. 2020;146:384–91.
36. Nordstrom A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, et al. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. *Proc Natl Acad Sci U S A*. 2004;101(21):8039–44.
37. Karakas FP, Turker AU. An efficient in vitro regeneration system for *Bellis perennis* L. and comparison of phenolic contents of field-grown and in vitro-grown leaves by LC-MS/MS. *Ind Crop Prod*. 2013;48:162–70.
38. Pirttila AM, Podolich O, Koskimaki JJ, Hohtola E, Hohtola A. Role of origin and endophyte infection in browning of bud-derived tissue cultures of Scots pine (*Pinus sylvestris* L.). *Plant Cell Tissue Organ Cult*. 2008;95(1):47–55.
39. Khan T, Abbasi BH, Iqar I, Khan MA, Shinwari ZK. Molecular identification and control of endophytic contamination during in vitro plantlet development of *Fagonia indica*. *Acta Physiol Plant*. 2018;40(8):9.
40. Fang JY, Hsu YR. Molecular identification and antibiotic control of endophytic bacterial contaminants from micropropagated *Aglaonema* cultures. *Plant Cell Tissue Organ Cult*. 2012;110(1):53–62.
41. Gwon SH, Heo JY, Kim BS. Identification and Control of Microbial Contaminants during in vitro Culturing of 'Atlantic' Potatoes. *Hortic Sci Technol*. 2019;37(4):528–39.
42. Han H, Liao Q, Marbaha W, Wang H, Zhuang H, Wang Q, et al. Isolation, Identification and Screening of Endophyte Fungicides for Disease Control during Tissue Culture of German Irises. *Xinjiang*

Figures

Figure 1

Calli induced from hypocotyl. (A) Calli induced from hypocotyls at 45 days; (B) callus observed under stereomicroscope.

Figure 2

Single-factor analysis of the impact of PGRs on the hypocotyl callus induction rate. Each value represents the mean $\pm$ SE of three independent experiments.

Figure 3

Effects of different concentrations of PGRs on the induction rate of root induced calli. Each value represents the mean $\pm$ SE of three independent experiments.

Figure 4

Representative images of the multiplication process and callus in different growth states. (A) Calli multiplication for 1 day; (B) calli multiplication for 45 days; (C) waterlogged callus; (D) callus in good condition; (E) callus with differentiation trend; (F) callus infected by endophytes; (G) callus infected by endophyte leading to death; (H) browning callus.

Figure 5

Single-factor analysis of the effect of PGRs on the differentiation rate of root induced calli. Each value represents the mean $\pm$ SE of three independent experiments.

Figure 6

Impact of antibiotic concentrations on endophyte inhibition and browning rates for calli. (A) Effect of three antibiotics on endophyte inhibition rates for calli; (B) effect of three antibiotics on the browning rate of calli; (C) effect of penicillin G concentrations on endophyte inhibition and browning rates.

Figure 7

Root induced calli under stereomicroscopy. (A) Callus induction at one end of root at the incision site; (B) the incision site where the fibrous roots were cut off.