



Investigating the relationships between callus browning in *Isatis indigotica* Fortune, total phenol content, and PPO and POD activities

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Received: 10 June 2023 / Accepted: 5 July 2023 / Published online: 20 July 2023
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Abstract

Browning, as the most important problem to be solved in callus differentiation, occupies an important position in tissue culture. In this study, plant regeneration was induced using leaves from germinated *Isatis indigotica* Fortune seedlings and mature plants as explants. Activated carbon (AC) and a solidification agent (Phytigel) were added simultaneously to inhibit browning. We measured the total phenol content and the PPO and POD activities in callus-induced seedling leaves and mature plant leaves of different ploidy at various stages. The results demonstrated that single plant growth regulators could induce calli with higher rooting and browning rates. Despite the addition of AC and phytigel to the medium, half of the mature plant calli turned yellow and eventually died. The total phenol content and POD activity in calli from mature leaves showed significant differences within 0–4 weeks ($P < 0.05$) but were still higher than the biochemical parameters in calli induced from seedlings, while the PPO activity did not differ significantly ($P > 0.05$). Mature materials of different ploidy yielded similar results. The addition of 2 mg·L⁻¹ BAP, 0.1 mg·L⁻¹ NAA, and 1.5 mg·L⁻¹ AC and solidification with 0.2% phytigel in MS medium resulted in the highest callus regeneration rate and the lowest browning rate. During dedifferentiation, the browning of the two may be associated with PPO activity and total phenol content.

Key message

The regeneration system of *Isatis indigotica* Fortune was optimized and the browning problem was solved. The browning of callus is related to PPO activity and the total phenol content.

Keywords *Isatis indigotica* Fortune · Callus · Browning · PPO · POD · Total phenol content

Abbreviations

<i>Isatis indigotica</i> Fortune	<i>I. indigotica</i>	2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-benzylaminopurine	KT	Kinetin
NAA	1-naphthylacetic acid	MS	Murashige and skoog
		CIM	Callus induction medium

Communicated by Christell van der Vyver.

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PPO	Polyphenol oxidase
POD	Peroxidase
AC	Activated carbon
ASA	Ascorbic acid
D	Day
H	Hour
Min	Minute

Introduction

Isatis indigotica Fortune is a cruciferous herb. Its root is *Isatidis Radix* (Banlangen in Chinese), its leaf is *Isatidis Folium* (Daqingye in Chinese) (Chen et al. 2014), and it is also the original plant of indigo naturalis (Qing dai in Chinese) (Wang 2018). The primary active compounds isolated from this species comprise terpenoids, lignans, and indole alkaloids. These compounds have proven antiviral, antibacterial, anti-inflammatory, and antileukaemia properties (Kang et al. 2020). *I. indigotica* has been used as an important medicinal plant, the therapeutic amount required is relatively large, and demand exceeds supply. Effective prescriptions screened by traditional Chinese medicine to treat *COVID-19* include “Three medicines and three prescriptions,” of which *Isatidis Radix* is the main component of the “Lianhuaqingwen capsule” (Xiao et al. 2020).

The leaves, roots, and hypocotyls of *I. indigotica* are often used to induce calli (Ke et al. 2009), and the induction rate of different parts is also different (Chen et al. 2015). Although the induced differentiation rate is high, browning is the most common problem. In recent years, many scholars have carried out extensive research on browning. These researchers believe that plant browning is caused by the action of oxidases, namely, polyphenol oxidase and peroxidase, including polyphenol oxidase (PPO) and peroxidase (POD), on natural substrate phenols (Sharma et al. 2019). In normal plant tissues, the simultaneous presence of substrate, oxygen, and PPO does not cause browning because polyphenols are distributed in vacuoles in normal tissue cells, and enzymes are distributed in various plastids and the cytoplasm. This regional distribution prevents the substrate from contacting polyphenols, thus avoiding the occurrence of enzymatic browning in normal tissues (Koochi et al. 2020). Phenols participate in respiratory metabolism as respiratory transmitters in normal plant tissues, and the redox between phenols and quinones is in dynamic balance. When plant tissue cells are damaged, the typical respiratory chain is interrupted, and the invasion of oxygen causes phenolic substances to rapidly oxidize to *O*-quinones under the action of oxidase and then form brown pigment or melanin through rapid nonenzymatic polymerization (Gong et al. 2015). As the most challenging problem to be solved

in the regeneration system, there is an urgent need to solve the browning problem to improve the value-added rate of plants to accumulate secondary metabolites and characterize genes.

In this study, different concentrations of plant growth regulators (PGRs) were used to induce the dedifferentiation of the young leaves of seedlings germinated from *I. indigotica* seeds and the leaves of mature plants to form a callus and then to differentiate into seedlings. The effects of different antibrowning inhibitors on the induction and regeneration of calli were evaluated, and an effective high-frequency transformation method for indigo was established on this basis. This method can promote the breeding of excellent varieties of *I. indigotica* and effectively protect wild *I. indigotica* resources.

Materials and methods

Materials and conditions

Diploid seeds of *I. indigotica* (Baoding, Hebei) and tetraploid seeds of *I. indigotica* (Qianxian, Shaanxi) were supplied by Resources Sanjiu Medical & Pharmaceutical Co., Ltd. of China. Dr. Qiaosheng Guo of Nanjing Agricultural University identified the seeds. Mature *I. indigotica* plants (1 year old), including *I. indigotica* (Baoding, Hebei) and *I. indigotica* (Qianxian, Shaanxi), were planted at the Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing, Jiangsu Province (32°2'6.25"N, 118°50'23.47"E, altitude, 50.21 m). The voucher specimen was kept at the Institute of Chinese Medicinal Materials, Nanjing Agricultural University.

Duan's method was used to disinfect the seeds and mature plant leaves and conduct differentiation in Murashige and Skoog (MS) medium with different concentrations of PGRs (Duan et al. 2019). The MS medium contained 3% ($w \cdot v^{-1}$) sucrose and 0.7% agar. The pH of the medium was 5.8–6.0, and the mixture was autoclaved at 121 °C for 20 min. Sucrose was purchased from China Guanghua Co., Ltd. MS medium and agar were purchased from Beijing Solarbio Technology Co., Ltd.

Callus induction

The leaves were from 14 d seedlings, and mature plants were cut into approximately 1 cm² pieces with a blade and placed on callus induction medium (CIM). The medium contained 2,4-dichlorophenoxyacetic acid (2,4-D; 2, 1 or 0.1 mg·L⁻¹), 1-naphthylacetic acid (NAA; 2, 1 or 0.1 mg·L⁻¹), kinetin (KT; 2, 1 or 0.1 mg·L⁻¹) and 6-benzylaminopurine (BAP;

2, 1 or 0.1 mg·L⁻¹). After four weeks of culture, the quantity of callus generated through dedifferentiation was recorded.

Antibrowning treatments

The calli browned in the process of redifferentiation. We added activated carbon (AC) and phytigel to the medium antibrowning treatment. The MS medium used contains 0.00001% copper sorbitol, 0.5% sulfate, 0.7% agar or 0.2% phytigel, and AC. At the same time, 2 mg·L⁻¹ BAP and 0.1 mg·L⁻¹ NAA were added for culture. Leaves were placed on different treatment media, and the browning percentage was recorded over two weeks.

Redifferentiation

The experiment involved optimizing the medium using various proportions of auxin and cytokinin. Select the optimal treatment among antibrowning treatments to apply to the redifferentiation. The medium contained 0.00001% copper sorbitol, 0.5% sulfate, and different concentrations of BAP and NAA, i.e., high, middle, and low concentrations of 2, 1, and 0.1 mg·L⁻¹, respectively. After four weeks, the percentage of differentiated calli was determined. The buds differentiated from the callus will continue to grow in the differentiation medium. after they grew to 2 ~ 5 cm, they were transferred to 1/2 MS and allowed to take root. After one month of cultivation, the rooted seedlings were transferred to flower pots and grown in a greenhouse (25 ± 1 °C).

Determination of biochemical parameters

The physiological indices of calli in redifferentiation medium were determined, including the total phenol content and activities of PPO and POD. Calli from 5 periods (0, 1, 2, 3, 4 weeks) were collected and dried at room temperature (20 ± 1 °C) and then ground into a fine powder. The dried samples were passed through a 100-mesh sieve and stored in a refrigerator at 4 °C. One gram of sample was weighed, and 3 mL of methanol was used for ultrasonic extraction for 60 min, followed by determination by spectrophotometry (Alpha-1506, Ltd., Shanghai, China). The total phenol content was determined by Folin–Ciocalteu colorimetry at OD₇₂₅.

The activity of PPO was determined by spectrophotometry with catechol solution as the substrate. The results are expressed as OD₅₂₅ min⁻¹ g⁻¹ of protein. The activity of POD was determined by spectrophotometry using guaiacol and H₂O₂ solution as substrates. The results are expressed as OD₄₇₀ min⁻¹ g⁻¹ protein (Yang et al. 2009).

Statistical analysis

SPSS 23.0 was used for statistical analysis. Analysis of variance and Duncan's multiple range test were used for significance analysis of the data.

Results

Induction of calli from various parts of *I. indigotica*

To identify the most efficient induction method, the leaves of seedlings and mature plants were treated (Fig. 1a, d). The leaves were transferred to CIM with BAP, NAA, 2,4-D, and KT, as listed in Table 1. Different hormones in the medium could induce calli, and the induction rate was 53 ~ 96%. BAP and NAA played an active role in the induction process. When the concentrations of BAP and NAA were 2 mg·L⁻¹, the induction rates were over 90%. Calli appeared around the explants after 10 d of the culture-inducing medium. Figure 1b, e shows the calli of *I. indigotica* (Baoding, Hebei) growing around the perimeter after two weeks.

Treatment with antibrowning agents in different media

After incubation in MS medium supplemented with 0.7% agar, more than 70% of the mature explants were browned, and the seedlings were not all healthy (Fig. 1h). After adding AC and phytigel to the MS medium, 50% of the mature calli developed well, while 88–93% of the seedling calli grew without browning.

Different antibrowning agents were selected to study the difference in the callus differentiation rate between mature plants and seedlings. In other media, phytigel had a better antibrowning rate than agar, while AC did not differ significantly. Seedling calli were inoculated on regeneration medium with 2.0 g·L⁻¹ phytigel. With 1.5 g·L⁻¹ AC, browning started after 21 d, 12 d later than a mature callus. The callus differentiation in mature plants increased from 33 to 50%, but half were unhealthy. The best antibrowning medium included 1.5 g·L⁻¹ AC solidified with 0.2% phytigel in MS medium, and the browning rate and rooting rate were the lowest under this condition (Table 2).

Callus redifferentiation to form seedlings

Although seedlings and mature explants could dedifferentiate to form calli, the ability to redifferentiate into shoots was quite different. Calli from the leaves of mature plants were over half browned and eventually died after two weeks (Fig. 1e, f). After 14 d, calli from seedlings redifferentiated

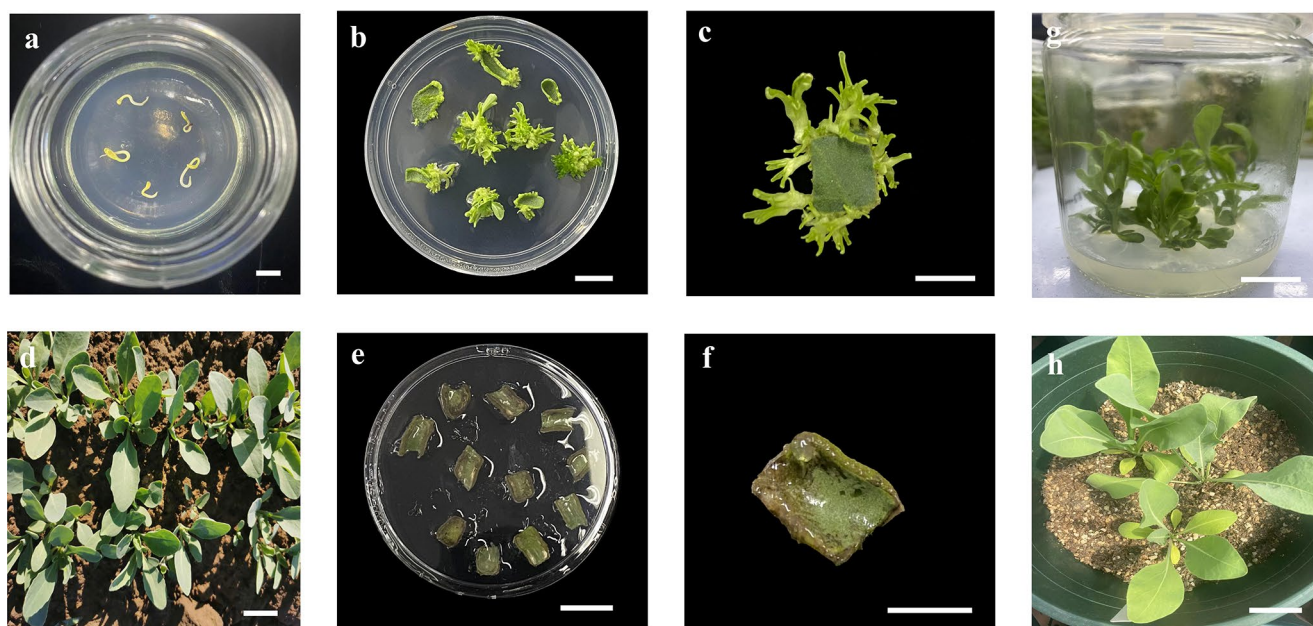


Fig. 1 Callus-mediated plant regeneration in *I. indigotica* (Baoding, Hebei). **a**. Seven-day-old seedlings; **b**. Callus induction from the leaf tissue of seedlings on CIM for 3 weeks; **c**. A regenerated plantlet from a seedling-derived callus; **d**. Field-grown mature plants with young leaves used as explants; **e**. Callus induction from leaf tissue of

field-grown mature plants on CIM for 3 weeks; **f**. Mature plant-derived calli on regeneration medium for 3 weeks; **g**. Rooting of a plantlet for 3 weeks; **h**. A regenerated plant transplanted to soil for 4 weeks Bars = 1 cm

Table 1 Effect of plant growth regulators on the callus initiation rate of leaf explants from seedlings and mature *I. indigotica* plants

PGR concentration (mg·L ⁻¹)		Mature plants (%)	Seedlings (%)
NAA	0.1	84.6 ± 4.5 ^b	87.6 ± 2.4 ^b
NAA	1	92.9 ± 3.1 ^a	93.5 ± 3.1 ^a
NAA	2	96.3 ± 5.2 ^a	95.5 ± 3.2 ^a
BAP	0.1	85.7 ± 6.2 ^b	87.4 ± 4.5 ^b
BAP	1	82.2 ± 3.8 ^b	84.8 ± 5.6 ^b
BAP	2	92.7 ± 3.7 ^a	93.5 ± 3.5 ^a
KT	0.1	75.7 ± 4.4 ^c	69.4 ± 2.1 ^c
KT	1	72.3 ± 4.2 ^c	85.7 ± 1.7 ^b
KT	2	68.2 ± 6.1 ^d	73.2 ± 3.6 ^c
2,4-D	0.1	53.2 ± 5.2 ^e	65.2 ± 3.2 ^d
2,4-D	1	61.7 ± 4.3 ^d	63.5 ± 2.4 ^d
2,4-D	2	55.6 ± 5.2 ^e	60.2 ± 4.5 ^e

Data are presented as the means ± standard deviations from three replicates with 60 explants in each replicate. Values within a column followed by a different letter are significantly different at $P < 0.05$ according to Duncan's multiple range test

PGR plant growth regulator, BAP 6-benzylaminopurine, NAA 1-naphthyleacetic acid, KT Kinetin, 2,4-D 2,4-Dichlorophenoxyacetic acid

into form buds (Fig. 1b, c). Combining BAP and NAA, two-factor induction was used to screen the dedifferentiation medium. The results showed that when 2 mg·L⁻¹ BAP and 0.1 mg·L⁻¹ NAA were added to the MS medium, the differentiation rate was the highest, reaching 85.8%. The rooting

rate was 0%, and the differentiation rate was the highest (Table 3).

When the seedlings grew to 3 ~ 5 cm on the redifferentiation medium, the seedlings were cut and inoculated into 1/2 MS medium. After approximately 18 d, the seedlings developed roots (Fig. 1g). After opening the sealing film on the medium, cultivating the seedlings for one week, and then transferring them to potting soil (Fig. 1h), a higher survival rate could be obtained.

Comparative analysis of the total phenol content and the PPO and POD activities in different materials

Browning is the most common problem when growing in medium (Tang et al. 2004). When the growth reaches a particular stage, it will turn black, affecting the callus's growth and development. Studies have shown that these browning phenomena are usually related to the total phenol content and oxidase activity (PPO and POD). Plants of different ploidy in experiments demonstrated the same trend. The total phenol content and the PPO and POD activities in calluses induced from seed-derived seedlings was lower than those in calluses induced from mature plants.

The total phenol content and the activities of PPO and POD all declined in diploids (*I. indigotica*, Baoding) and tetraploids (*I. indigotica*, Xianxian) during the differentiation culture period. As time passed, the decreases observed

Table 2 Percentage of nonbrowned calli during differentiation with antibrowning treatments in *I. indigotica*

Treatments	Mature plant		Seedling	
	Nonbrowned Calli (%)	Days of browning (Day)	Nonbrowned Calli (%)	Days of browning (Day)
0.7% agar	33.3 ± 0.5 ^d	9	85.7 ± 0.5 ^b	19
0.7% agar + 0.15% AC	35.5 ± 2.4 ^d	9	88.9 ± 1.4 ^b	19
0.2% phytigel	45.5 ± 2.7 ^c	11	90.1 ± 1.2 ^a	21
0.2% phytigel + 0.15% AC	50.0 ± 3.5 ^c	11	93.7 ± 1.5 ^a	21

The data are presented as the means ± standard deviations from three replicates with 60 explants in each replicate. Values within a column followed by a different letter are significantly different at $P < 0.05$ according to Duncan's multiple range test

AC activated carbon

Table 3 Effect of plant growth regulators on shoot formation from seedling-derived calli

PGR concentration (mg·L ⁻¹)		% of callus differentiating plants	% of rooting
BAP	NAA		
2	2	80.6 ± 6.2 ^b	14.93 ± 3.8 ^d
2	1	58.3 ± 6.7 ^d	0
2	0.1	85.8 ± 5.5 ^b	0
1	2	88.2 ± 3.2 ^a	23.4 ± 2.4 ^c
1	1	71.6 ± 5.7 ^c	22.2 ± 3.4 ^c
1	0.1	83.5 ± 5.5 ^b	6.7 ± 3.7 ^e
0.1	2	86.9 ± 2.5 ^b	4.78 ± 2.3 ^e
0.1	1	91.6 ± 3.5 ^a	91.2 ± 8.2 ^a
0.1	0.1	71.4 ± 10.5 ^c	35.7 ± 6.3 ^b

The data are presented as the means ± standard deviations from three replicates with 60 explants in each replicate. Values within a column followed by a different letter are significantly different at $P < 0.05$ according to Duncan's multiple range test

PGR plant growth regulator, BAP 6-benzylaminopurine, NAA 1-naphthaleneacetic acid

in calli derived from seedlings were higher than those of calli induced from mature plants. After four weeks, the total phenol content and the activities of POD and PPO in the calli derived from diploid seedlings decreased by 53.4%, 42.2%, and 65.9%, respectively. The calli derived from the mature diploid leaves decreased by 42.6%, 49.3%, and 30.7%. The total phenol content and PPO activity of calli derived from diploid seedlings were significantly lower than those before four weeks.

In contrast, the POD activity did not change significantly but was still lower than that of calli derived from mature plant leaf tissue. The tetraploid also showed the same trend

(Table 4). The differences in callus browning during culture might be related to the total phenol content and PPO activity.

Discussion

Optimization of plant growth regulators in the *I. indigotica* regeneration system

Establishing an efficient, rapid, and stable genetic transformation system is essential to validate candidate genes (Abdallat et al. 2011). With the gradual modernization of traditional Chinese medicine, plant tissue culture technology can rapidly reproduce plant tissue in vitro in a short period (Sundararajan et al. 2022). This method is fast, efficient, controllable and convenient for large-scale industrial production. Callus-mediated transformation is the most common method of obtaining transgenic plants (Xu et al. 2017). Reportedly, the stems, hypocotyls, true leaves, cotyledons, and petioles of watercress can induce a callus, and its leaves and stems have higher induction rates (Li et al. 2023). Callus induction experiments in *Erysimum scoparium* (Cruciferous) explants cultured in MS medium showed that the leaves and hypocotyls have a high induction rate, while the roots have a low induction rate. It has also been reported that the leaves of 10- to 14-d-old sterile cauliflower seedlings are better than 15- to 20-d-old leaves. Moreover, the differentiation and induction rates of protoplasts are higher. The vacuoles are small and dispersed, and the chloroplasts are uniformly distributed (Gerszberg 2018). Consequently, leaves are the best material for inducing calli, and their cell totipotency determines the differentiated shoots. In *Narcissus confusus* (Amaryllidaceae), calli induced by sterile seedlings cultured from seed had a high regeneration capacity. Moreover, all regenerated plants showed typical morphological characteristics (Sellés et al. 1999; Santos et al. 2020). Using sterile seedling explants to induce

Table 4 Differences in polyphenol content and POD and PPO activities

Explants	Polyphenols (mg·g ⁻¹)	POD (OD ₄₇₀ min ⁻¹ g ⁻¹ protein)	PPO (OD ₅₂₅ min ⁻¹ g ⁻¹ protein)
Mature plant in <i>I. indigotica</i> (Baoding)			
0 week	2.72 ± 0.25 ^a	3.00 ± 0.36 ^a	2.05 ± 0.35 ^b
1 week	1.20 ± 0.15 ^b	2.06 ± 0.35 ^b	2.04 ± 0.29 ^b
2 week	1.99 ± 0.34 ^b	2.04 ± 0.27 ^b	1.92 ± 0.28 ^b
3 week	1.59 ± 0.23 ^b	1.84 ± 0.27 ^b	1.43 ± 0.19 ^b
4 week	1.56 ± 0.14 ^b	1.52 ± 0.18 ^b	1.42 ± 0.15 ^b
Seedling in <i>I. indigotica</i> (Baoding)			
0 week	2.32 ± 0.35 ^{ab}	2.25 ± 0.45 ^{ab}	2.76 ± 0.38 ^a
1 week	1.92 ± 0.24 ^b	1.95 ± 0.36 ^b	1.52 ± 0.28 ^b
2 week	1.91 ± 0.21 ^b	1.50 ± 0.22 ^b	1.66 ± 0.21 ^b
3 week	2.01 ± 0.18 ^b	1.52 ± 0.12 ^b	1.34 ± 0.15 ^b
4 week	1.08 ± 0.12 ^{bc}	1.30 ± 0.08 ^b	0.94 ± 0.09 ^c
Mature plant in <i>I. indigotica</i> (Qianxian)			
0 week	2.85 ± 0.37 ^a	2.70 ± 0.41 ^a	2.31 ± 0.45 ^{ab}
1 week	1.98 ± 0.25 ^b	1.90 ± 0.38 ^b	1.77 ± 0.16 ^b
2 week	1.90 ± 0.27 ^b	2.00 ± 0.28 ^b	1.65 ± 0.15 ^b
3 week	1.68 ± 0.17 ^b	1.54 ± 0.13 ^b	1.40 ± 0.19 ^b
4 week	1.65 ± 0.15 ^b	1.53 ± 0.15 ^b	1.36 ± 0.12 ^b
Seedling in <i>I. indigotica</i> (Qianxian)			
0 week	1.99 ± 0.39 ^b	2.01 ± 0.35 ^b	2.10 ± 0.35 ^b
1 week	1.85 ± 0.28 ^b	1.87 ± 0.29 ^b	2.07 ± 0.34 ^b
2 week	1.65 ± 0.24 ^b	1.34 ± 0.18 ^b	1.83 ± 0.15 ^b
3 week	1.51 ± 0.28 ^b	1.45 ± 0.15 ^b	0.94 ± 0.05 ^c
4 week	1.18 ± 0.12 ^{bc}	1.26 ± 0.08 ^b	0.85 ± 0.07 ^c

The data are presented as the means ± standard deviations from three replicates with 60 explants in each replicate. Values within a column followed by a different letter are significantly different at $P < 0.05$ according to Duncan's multiple range test

POD peroxidase, PPO polyphenol oxidase

differentiation for plant regeneration is ideal for cultivating directly inserted petiole cotyledons (Yao et al. 2011).

The commonly used exogenous plant growth substances can be divided into two categories, auxin and cytokinin. The combination of auxin and cytokinin in proportion can promote plant induction (stem induction, proliferation induction, callus induction, and tissue culture seedling rooting) (Meng et al. 2014). When the two are present in low amounts, the induction effect is not significant. Excessively high concentrations not only inhibit the formation of buds but also increase the differentiation of roots. He found that different plant growth substance combinations of 2,4-D, 6-BA, and NAA played a crucial role in *Brassica oleracea* var. albiflora Kuntze. The optimal induction medium was MS + 0.1 mg·L⁻¹ 2,4-D + 0.02 mg·L⁻¹ 6-BA, and the induction rate of hypocotyls was as high as 80%. The optimal medium for the regeneration culture process was MS + 0.75 mg·L⁻¹ 6-BA + 0.03 mg·L⁻¹ NAA, with the

adventitious bud differentiation rate and rooting rate reaching 94% and 67%, respectively (He et al. 2019). In general, 6-BA is used in the proliferation culture of the primary induction medium; NAA is used in the induction of callus, monocotyledonous plants require more hormone concentration than dicotyledonous plants, and induction rooting culture generally uses indole-3-butyric acid (IBA).

In this experiment, the PGR types and combinations of concentrations were intricate, different concentrations of a single PGR were used to induce calli, and the ratios of dual PGR combinations was optimized to induce calli to differentiate into shoots. In MS medium, different concentrations of PGRs, including BAP, NAA, 2,4-D, and KT, could cause calli at rates higher than 80% (Table 1). Both mature plants and 14-d-old seedlings can induce calli and redifferentiate into seedlings, and the differentiation rate of seedlings is higher than that of mature plants. Table 1 shows that NAA and BAP can induce higher levels of callus formation. Based on previous reports (He et al. 2019), the concentration range was determined to be 0.1 ~ 2 mg·L⁻¹. A combination of BAP and NAA was used to induce adventitious buds. The results showed that the callus induction rate was the highest under treatment with 2 mg·L⁻¹ BAP and 0.1 mg·L⁻¹ NAA, especially since its rooting rate was 0%. The differentiation rate of different explants exhibited significant disparity upon additional processing, as shown in Table 3.

Causes of browning in *I. indigotica*

Although the regeneration medium included browning inhibitors, calli derived from mature leaves still easily browned and were challenging to differentiate. We wanted to take measures to reduce browning to confirm the reasons for rooting and browning in mature explants. There are many ways to alleviate or delay the browning of explants in tissue culture, such as antioxidants (Aghayeh et al. 2021) and altering culture conditions such as the temperature, pH, moisture, and salinity stress (Ndakidemi et al. 2014). Duan added AC, ascorbic acid (ASA) and solidification agent to the medium and found that the nonbrowning rate of calli from seedlings in the regeneration medium containing phytigel and ASA was as high as 83.3%, which was consistent with previous results (Duan et al. 2019). The browning rate was reduced by changing the curing agent of the medium in this experiment. Compared with agar, phytigel significantly affected the callus, delaying the browning rate by 2 d and increasing the browning rate by 16.7% and 8%, respectively (Table 2). The results showed that adding phytigel to the medium could effectively alleviate the browning rate of *I. indigotica* calli.

Although phytigel was used in the regeneration medium, the calli induced from the leaves of mature plants were

mostly browned and not differentiated. Likewise, the leaves from young shoots germinated from *Isodon amethystoides* seeds induced a higher rate of callus differentiation compared with leaves from mature plants (Duan et al. 2019). This finding shows that the growth vigour of the leaves of young shoots germinated from seeds is higher than that of mature plants. At the same time, the oxidation reaction may also be another reason for its browning (Schuch et al. 2010).

However, polyphenols, as substrates for PPO and POD reactions, have been implicated as one of the factors affecting the browning of explants in tissue culture (Leng et al. 2009). The oxidation of phenolic compounds into their corresponding O-quinones catalysed by PPO is now well documented (Lim et al. 2019). PPO is also the major protease in plant tissue browning (Xu et al. 2015). Additionally, POD can oxidize various phenolic compounds, including monohydroxy and catholic phenolics (Soltani et al. 2023). PPO is responsible for the enzymatic browning of apples; apples lacking PPO activity might be helpful not only in the food industry but also in studies of the metabolism of polyphenols and the function of PPO (Wu et al. 2021). PPO and POD can convert phenolic compounds into O-quinones (Garcia et al. 2007). We determined the total phenol content and activities of PPO and POD in differentiating calli from seedling and mature plants at different ploidy levels. While POD activity exhibited an insignificant difference, the polyphenol content and PPO activity differed significantly. Both of them decreased in activities of PPO and total polyphenols. Materials with different ploidy levels demonstrated the same trend. These results are consistent with those for other plant species in previous reports. The activities of PPO and total polyphenols may be related to the browning of *I. indigotica* calli.

Conclusion

We optimized the *I. indigotica* plant regeneration system and selected the medium with the highest differentiation rate, i.e., $2 \text{ mg} \cdot \text{L}^{-1}$ BAP and $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA in MS medium. The solidification agent was optimized, and activated carbon was added to solve the browning problem. The total phenol content and the PPO and POD activities of materials of different ploidy were compared. The results showed that the browning rate was related to the PPO activity and the total phenolic substances. Using seedlings as starting materials, genetic transformation can lead to rapid differentiation with the lowest browning rate, which lays a foundation for subsequent gene mining.

Author contribution Conceived and designed the experiments: YS, MW, QG. Performed the experiments: YS, MW, QG. Analysed the data: YS, MW, QG, JH, KZ, JH. Wrote the paper: YS, MW, QG, JH,

KZ, JH.

Funding This work was supported by the Jiangsu Modern Agricultural Industrial Technology System Construction Project (JATS[2022]461, JATS[2022]291).

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

Declarations

Ethics approval and consent to participate We thank all authors for their contributions to this work.

Conflict of interest The authors declare that they have no conflicts of interest.

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