

# Establishment of *in vitro* regeneration system for leaves and petioles of *Oxalis triangularis* 'Purpurea'

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## Abstract

To provide good acceptor materials for genetic transformation, the leaves and petioles of *Oxalis triangularis* 'Purpurea' were used as explants to establish a tissue culture system including callus induction, subculture, bulb induction, rooting, and transplanting of seedlings. The results showed that the best callus induction explants were petioles. When they were inoculated to MS + 1.00 mg·L<sup>-1</sup> 6-BA + 0.50 mg·L<sup>-1</sup> NAA + 1.00 mg·L<sup>-1</sup> TDZ, granular callus induction rate was 53.33%. The optimal differentiation medium for the two different explants was MS + 1.00 mg·L<sup>-1</sup> 6-BA + 0.50 mg·L<sup>-1</sup> NAA. In this condition, petiole differentiation was 98.67% with an average differentiation coefficient of 11.19 in 45-d, and leaf differentiation was 97.38% with an average differentiation coefficient of 9.23 in 60-d. Four main types of healing tissues were produced during the induction of the two explants. The green granular callus and the green and purple loose callus were identified as embryonic callus. Villiform callus and green tight callus were identified as non-embryonic callus. The optimal subculture medium of embryonic callus was MS + 1.50 mg·L<sup>-1</sup> 6-BA + 0.20 mg·L<sup>-1</sup> NAA. When moving the adventitious buds to rooting medium, the optimal medium was 1/2 MS + 0.30 mg·L<sup>-1</sup> NAA + 0.30 mg·L<sup>-1</sup> IBA. In this condition, for 60 d, the number of adventitious buds, average root length, and plant height were 28.16 per plant, 1.96 cm and 6.62 cm respectively. The germination rate of sterile bulbs was as high as 100% after transplanting for 30 d. Flower buds could be formed and flowered on the 45<sup>th</sup> day of transplanting. This result provides technical support for the artificial large-scale propagation of *O. triangularis* 'Purpurea'.

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## Introduction

*Oxalis triangularis* 'Purpurea', a perennial herb belonging to Oxalidaceae is native to South America. It has palmately compound leaves, containing three inverted triangular leaflets with entire margins. The leaves are purplish red and cluster at the base<sup>[1,2]</sup>. *O. triangularis* 'Purpurea' is widely cultivated as a foliage potted flower, flower bed border plants, and garden ground cover ornamental plants. It is also a herbaceous plant with antibacterial, antifungal, and anticancer potential<sup>[3]</sup>. Its flowers show the characteristic of heterostyly and self-incompatibility. It cannot produce seeds by self-crossing but can only reproduce asexually by subterranean bulbs<sup>[4]</sup>. Therefore, it is difficult to obtain excellent varieties quickly by conventional breeding. Transgenic technology is an effective alternative to modifying horticultural flower traits by introducing other good trait genes. It is also an effective way to accelerate the genetic improvement of garden flowers<sup>[5]</sup>. However, the research on the genetic transformation of *O. triangularis* 'Purpurea' is still in the preliminary stage. It is far from satisfying the requirements of genetic improvement technology and commercial production.

*In vitro* culture is conducive to expanding production scale, improving plant quality, promoting germplasm improvement, and providing stable plant materials for genetic transformation

and gene editing technologies<sup>[6]</sup>. For the breeding of new varieties, the tissue culture of plants makes it easier to separate mutations<sup>[7]</sup>. There are still problems in studying rapid propagation of *O. triangularis* 'Purpurea' *in vitro*. For example, the callus induction rate and the number of induced cluster buds are low. The growth rate is also slow. Callus is an ideal transgenic material. It has the advantages of stable proliferation, high regeneration rate, and agrobacterium resistance<sup>[8]</sup>. At present, studies on *in vitro* culture of *Asparagus officinalis*<sup>[9]</sup>, *Dendrobium wilsonii*<sup>[10]</sup>, *Zingiber officinale*<sup>[11]</sup> and other plants have been reported. There have been studies on the *in vitro* flowering technology of *Oxalis* L. Prasuna et al. used *Oxalis corniculata* as material for tissue culture *in vitro*, and found that plant growth regulators, sucrose, MS, and light conditions could transform plants from vegetative growth to reproductive growth<sup>[12]</sup>. In addition to commonly used nutrients, the addition of TDZ to the medium can induce the proliferation of plant shoots in tissue culture<sup>[13–15]</sup>. However, there are few studies on embryonic callus of *O. triangularis* 'Purpurea', and the specific culture method is still unclear.

To explore the culture scheme of *O. triangularis* 'Purpurea' *in vitro*, the petiole and leaf of the plant were used as explants. The basic medium with 6-BA and NAA was supplemented with additional TDZ in different concentrations for callus induction, proliferation, adventitious bud rooting, and bulb induction. An

*in vitro* regeneration system of *O. triangularis* 'Purpurea' can be established to promote the development of transgenic plants. Meanwhile, it provides a basis for gene function study and has important reference significance for the induction of embryonic callus and genetic engineering breeding of other plants belonging to *Oxalis*.

## Materials and methods

### Materials

In the medium containing NAA and 6-BA, using petioles as explants could effectively improve the culture efficiency of the sterile plants of *O. triangularis* 'Purpurea'<sup>[16]</sup>. The study selected the tender petioles of *O. triangularis* 'Purpurea' with good greenhouse growth status as experimental materials. Petioles were rinsed in water for 3 h. On the ultra-clean bench, they were rinsed with 75% alcohol for 45 s and cleaned with sterile water for 1 min. Then petioles were sterilized with 3% explants disinfectant (Cygnus) oscillating for 8 min and washed twice with sterile water for 1 min each time. After cleaning, sterile filter paper was used to absorb moisture from the petioles surface. The petioles were cut into  $0.50 \pm 0.05$  cm and inoculated into MS medium containing  $1.00 \text{ mg}\cdot\text{L}^{-1}$  6-BA +  $0.50 \text{ mg}\cdot\text{L}^{-1}$  NAA +  $30 \text{ g}\cdot\text{L}^{-1}$  sucrose +  $7 \text{ g}\cdot\text{L}^{-1}$  agar +  $0.05 \text{ g}\cdot\text{L}^{-1}$  activated carbon (pH 5.7–5.8), which medium is hereinafter referred to as M1 medium. They were then cultured under the conditions of  $12 \text{ h}\cdot\text{d}^{-1}$  light cycle, 2,500–3,000 Lx light intensity,  $25 \pm 2$  °C and 50%–70% relative air humidity. The petioles were induced to differentiate into sterile plants and prepared for use. Robust and consistent tube seedlings were removed and placed on the ultra-clean bench. Leaves were cut into small square pieces about  $0.50 \text{ cm} \times 0.50 \text{ cm}$  and the petioles were cut into small segments  $0.50 \pm 0.05$  cm long. They were used as callus induction materials.

### Effects of different TDZ concentrations on callus induction of leaves and petioles and differentiation of adventitious buds

Sterile leaves and petioles were cut and placed on M1 medium with different TDZ concentrations (0.00, 0.50, 1.00, 1.50, 2.00, and  $2.50 \text{ mg}\cdot\text{L}^{-1}$ ). Five bottles were set in a treatment and each bottle had six explants. Each treatment was repeated three times. A total of 90 explants were included in this experiment. The six experimental groups were named T1–T6. Calli were observed every 5 d after inoculation. The first induction time and color of the petiole calli were recorded. The status, induction rate, differentiation rate and differentiation coefficient of granular calli were observed and recorded within 60 d.

### Types and morphological characteristics of callus

In the T1–T6 tests, calli induced by leaves and petioles at different stages (green loose, green granular, green compact,

and purple loose) and calli proliferating (white villous, purple loose) were observed and photographed under an NScope 2.0 stereoscopic microscope. The conventional paraffin section method adopted by Lian et al.<sup>[17]</sup> was used as a reference in this experiment. The calli of different periods were cut, fixed, dehydrated, impregnated with paraffin, embedded, and sliced. They were stained by hematoxylin-eosin staining. The images were then observed under an optical microscope (Nikon, Japan). After the sections were stained with hematoxylin and eosin, the nucleus was blue and the cytoplasm was red. According to morphological and cytological characteristics of callus, the characteristics of embryogenic callus were analyzed and identified.

### Callus proliferation

The induced calli with consistent growth status were placed on an ultra-clean bench. They were cut into the pieces of  $0.5 \text{ cm} \times 0.5 \text{ cm} \times 0.5 \text{ cm}$ . Then MS medium with different concentrations of NAA ( $0.20, 0.40 \text{ mg}\cdot\text{L}^{-1}$ ) and 6-BA ( $0.50, 1.50, 2.50 \text{ mg}\cdot\text{L}^{-1}$ ) were used for culture. The control group was MS medium without adding any hormone (named CK). The six experimental groups were named D1–D6. Three bottles were set in a treatment and each bottle had ten explants. Each treatment was repeated three times. Biomass gain multiple and callus multiplier multiple were measured after 30 d.

### Screening rooting medium

The adventitious buds with consistent growth status were placed on MS medium with different concentrations of NAA ( $0.00, 0.30, 0.50 \text{ mg}\cdot\text{L}^{-1}$ ) and IBA ( $0.00, 0.10, 0.30 \text{ mg}\cdot\text{L}^{-1}$ ). Ten bottles were set in a treatment and each bottle had six explants. Each treatment was repeated three times. The average bulb quantity, rooting rate, average number of roots, and average root length were counted after 60 d. The measurements were calculated after measuring with a centimeter scale ruler.

### Transplanting small bulbs

The small bulbs were induced in the rooting medium and removed from the culture bottles with a tweezer. The medium from the bulb surface was washed by running water. 75% wettable carbendazim powder and water were prepared into a solution at 1:1,000 and bulbs were soaked for 3 h. Finally, bulbs were transplanted into the substrate (peat soil : vermiculite = 3:1). Each pot was set to six bulbs. They were watered thoroughly after transplanting and conventional water management was carried out. The germination rate was measured every 10 d. A total of three observations were made.

### Data analysis

Excel and SPSS 20.0 software were used for the statistical analysis of the data. Duncan's new multiple-range test was used for the difference-significant analysis. Relevant indicators were calculated according to the following formula (1)–(10).

$$\text{Callus induction rate} = \frac{\text{Total number of callus induction}}{\text{Total number of calli in each group}} \times 100\% \quad (1)$$

$$\text{Differentiation rate} = \frac{\text{Total number of differentiated callus in each group}}{\text{Total number of petioles inoculated in each group}} \times 100\% \quad (2)$$

$$\text{Differentiation coefficient} = \frac{\text{Total number of differentiated adventitious buds of callus in each group}}{\text{Total number of differentiated calli in each group}} \quad (3)$$

$$\text{Biomass gain multiple} = \frac{\text{The weight of the callus mass in each bottle}}{\text{The weight of callus in each bottle at inoculation}} \quad (4)$$

$$\text{Callus multiplier multiple} = \frac{\text{The weight of the callus mass in each bottle (Callus weight after adventitious bud removal)}}{\text{The weight of callus in each bottle at inoculation}} \quad (5)$$

$$\text{Rooting rate} = \frac{\text{Number of roots in each bottle}}{\text{Number of inoculations in each bottle}} \times 100\% \quad (6)$$

$$\text{Average number of roots} = \frac{\text{Number of roots in each bottle}}{\text{Number of inoculations in each bottle}} \quad (7)$$

$$\text{Average root length} = \frac{\text{The length of the root in each bottle}}{\text{Number of inoculations in each bottle}} \quad (8)$$

$$\text{Average bulb quantity} = \frac{\text{Total number of bulbs induced in each bottle}}{\text{Number of adventitious buds inoculated in each bottle}} \quad (9)$$

$$\text{Bulb germination rate} = \frac{\text{Number of bulb sprouts}}{\text{Total number of seeded bulbs}} \times 100\% \quad (10)$$

## Results

### Effects of different TDZ concentrations on callus induction and differentiation of petioles

Previous experiments showed that petioles began to induce calli at 10 d in the TDZ treatment group. Calli were induced in all petioles at 12 d. Within 60 d, the calli had five states (Fig. 1a–f): white loose, green loose, purple loose, green granular, and green tight. These five states changed in sequence over time. Firstly, the petioles were induced into white loose calli. Then they turned into green and purple loose calli. Finally, some of them were induced into granular calli (Fig. 1g–h). Compared to no TDZ addition, the callus induction effect in the TDZ-containing medium was better. The entire petioles were transformed into numerous calli. More embryogenic calli were induced in the shortest time. When the concentration of TDZ was 0.50 or 1.00 mg·L<sup>-1</sup>, the granulated callus induction rate was the highest, at 45.48% and 53.33%, respectively, and there was no significant difference between them (Table 1). The granular calli continued to grow on the original medium and began to differentiate into adventitious buds at 18 d. However, the differentiation rate and differentiation coefficient of the

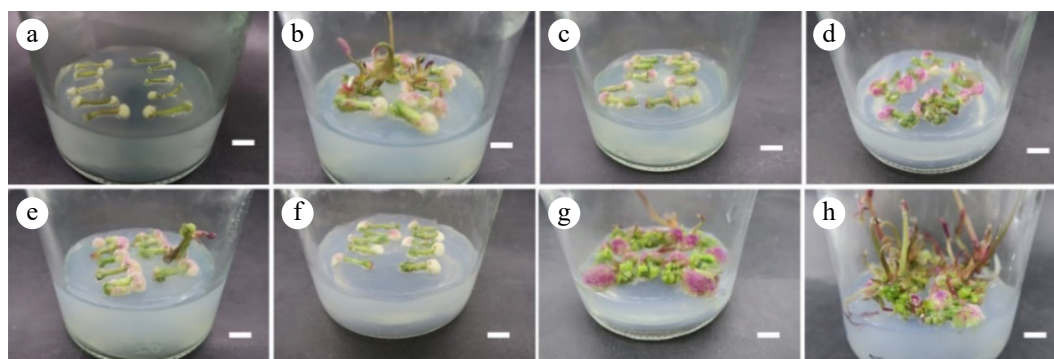
treatment group without TDZ were the highest, at 98.67% and 11.19, respectively.

### Effect of TDZ on calli induced by leaves of *O. triangularis* 'Purpurea'

The callus volume of leaves in the medium supplemented with TDZ was larger than that without TDZ (Fig. 2). Among them, 5% of the leaves induced granular-calli, and more than 95% of the calli were green loose. A large number of calli were generated around the wound throughout the leaves. In the medium supplemented with 0.50–2.50 mg·L<sup>-1</sup> TDZ, calli were induced in leaves within 15 d, and differentiation began on 24 d (Table 2). When TDZ concentration exceeded 0.5 mg·L<sup>-1</sup>, leaf differentiation was inhibited. The differentiation rate of leaf callus was less than 30% and the average differentiation coefficient was less than 5. In the control group, calli began to be induced on 24 d, and the differentiation rate reached 97.38%. The average differentiation coefficient of single leaf was 9.23 after 60 d.

### Morphological and histological observation of callus induced by different explants

It was found that the callus status of *O. triangularis* 'Purpurea' varied with time. The leaf organs were inoculated on



**Fig. 1** Callus cultivated from petiole. (a)–(f) Corresponding to the results of petiole induction callus in the T1–T6 group, respectively; (g), (h) Green granular callus. Scale bar = 1 cm.

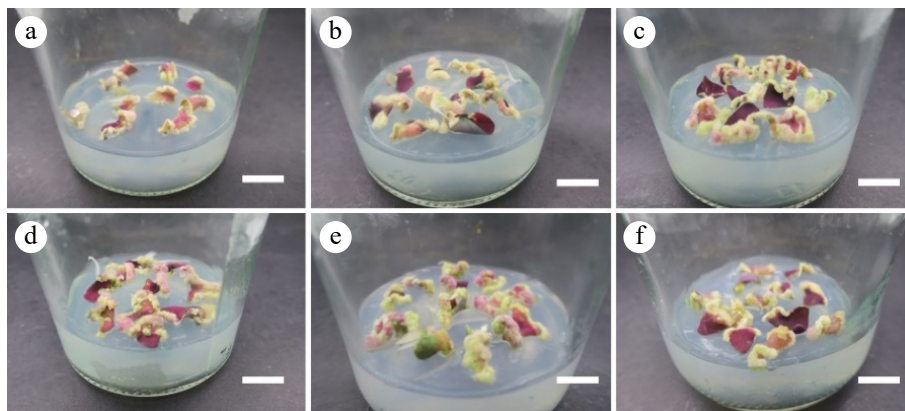
**Table 1.** Effects of different TDZ concentrations on callus induction and differentiation of petiole.

Treatment	TDZ concentrations (mg·L <sup>-1</sup> )	Callus status	Callus volume	Granular callus induction rate (%)	Differentiation rate/%	Differentiation coefficient
T1	0.00	White, loose	Only produced at both ends of the wound	15.43 ± 5.05de	98.67 ± 1.44a	11.19 ± 0.45a
T2	0.50	Purple, granular, loose	Produced at whole petioles	45.48 ± 5.07ab	65.00 ± 5.00b	3.19 ± 0.27b
T3	1.00	Purple, granular	Produced at whole petioles	53.33 ± 7.64a	58.33 ± 2.89c	3.06 ± 0.35b
T4	1.50	Purple, granular	Produced at whole petioles	32.87 ± 9.31bc	43.33 ± 0.06e	2.39 ± 0.32c
T5	2.00	Purple, granular, partial white loose	Produced at whole petioles	7.78 ± 7.69f	52.22 ± 1.93d	2.40 ± 0.18c
T6	2.50	Purple, granular, partial white loose	Produced at whole petioles	27.78 ± 1.92cd	52.22 ± 13.88d	3.39 ± 0.49b

The data are mean ± standard deviation. Different letters after the same column of numbers indicate significant differences between different treatments ( $p < 0.05$ ).

TDZ-containing medium for callus induction. The petioles formed the white granular calli on the 10<sup>th</sup> day (leaves on the 16<sup>th</sup> day) (Fig. 3a) and then became the green tight calli (Fig. 3b). At 20–40 d, calli began to turn into green granular calluses (Fig. 3c). When the calli were cultured on the same medium for more than 60 d, the loose embryogenic calli began to transform into tight embryogenic calli (Fig. 3d), which grew slowly and did not differentiate. Calli were divided into the following four types according to external morphology. Callus I was a white villous calli with a tight texture and was not easily broken when touched by tweezers. Callus II was a green loose calli with loose texture and green granular surface and was easy to damage when touched by tweezers. Callus III was purple and green loose calli with a soft texture and could be damaged when touched by tweezers. Callus IV was a tawny calli with a compact texture and fleshy inside and was easy to brown but not easy to damage when touched by tweezers.

Both cell morphologies are seen in Fig. 3. One type of cell was an embryogenic cell. It had the characteristics of small size, tight and neat arrangement, large oblong nucleus, dense cytoplasm, high karyoplasmic ratio, deep staining, and vigorous cell division. Most of these cells appeared at the edge of the callus and had cytoplasm. The other type was a nonembryonic cell, had the characteristics of large and different volume, irregular arrangement, small round nucleus in the center of the cell, thin cytoplasm, low karyoplasmic ratio, shallow staining, and poor cell differentiation ability. Most of these cells had no nucleus. From the perspective of histology and morphology, with leaves and petioles as explants, the white compact callus (Fig. 3e) initially induced on M1 medium was identified as a non-embryonic callus. The green, and purple loose callus and granular callus induced by TDZ were embryogenic (Fig. 3f–g). On the 90<sup>th</sup> day, callus cells became larger, and embryogenic cells were less. It grew at a slower rate and became less differentiated,

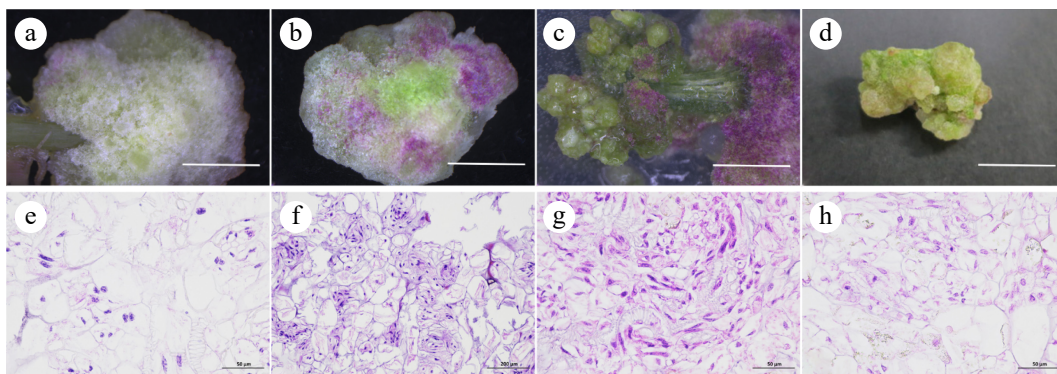


**Fig. 2** Calli cultivated from leaves. (a)–(f) Corresponding to the results of leaf induction callus in the T1–T6 group. Scale bar = 1 cm.

**Table 2.** Effects of different TDZ concentrations on callus induction and differentiation of leaf leaves.

Treatment	TDZ concentrations (mg·L <sup>-1</sup> )	Callus volume	Initial induction of callus (d)	Differentiation rate (%)	Differentiation coefficient
T1	0.00	Only produced around the wound	24	97.38 ± 2.27a	9.23 ± 0.64a
T2	0.50	Produced at whole leaves	15	25.56 ± 7.70b	4.26 ± 0.52bc
T3	1.00	Produced at whole leaves	15	11.11 ± 1.93cd	4.33 ± 2.75bc
T4	1.50	Produced at whole leaves	15	8.33 ± 2.89d	1.5 ± 0.50c
T5	2.00	Produced at whole leaves	15	13.33 ± 7.64cd	5.33 ± 3.21b
T6	2.50	Produced at whole leaves	15	29.47 ± 32.21bc	2.67 ± 0.95bc

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ( $p < 0.05$ ).



**Fig. 3** Induction and histological observation of callus from petioles and leaves. (a), (e) Callus I. (b), (f) Callus II. (c), (g) Callus III. (d), (h) Callus IV, scale bar = 1 cm.

then gradually transformed into a non-embryonic callus (Fig. 3h).

### Effects of different 6-BA and NAA treatments on callus proliferation

To keep the calli in the embryonic stage as long as possible, the combination of 6-BA and NAA with different concentrations was set up to observe and analyze the proliferation of the callus. At 7 d, the calli had a few white villi growing on the outside. Part of the calluses in groups CK, D1, and D3 started browning and were unchanged in other groups. At 14 d, the calli began to grow significantly and many white calli grew on the surface of most calli. The proliferation rate of callus slowed down at 21 d. There was no significant change in callus proliferation at 21–30 d.

The medium combined with NAA (0.20, 0.40 mg·L<sup>-1</sup>) and 6-BA (0.50, 1.50, 2.50 mg·L<sup>-1</sup>) had no significant effect on proliferation and callus multiplier multiple. The change in biomass gain multiple was significant. In the D2 and D6 treatment groups, at 30 d, the biomass gain multiple of callus was the highest, at 4.23 and 4.05 times, respectively (Table 3). In the control group without any hormone, extensive browning occurred at the base of the calli (Fig. 4g). The biomass gains multiple and callus multiplier multiple were the lowest, at 1.86 and 1.93 times, respectively (Table 3).

### Effects of different concentrations of IBA on adventitious bud growth

In this study, the optimal growth conditions of adventitious buds were explored, and the effects of different IBA concentrations on biomass gain multiple and plant status were compared. In the M1 medium, with the increase of IBA concentration, the gain multiple of advent buds showed a trend of first increasing and then decreasing (Table 4). When the

concentration of IBA was 0.3 mg·L<sup>-1</sup>, the advent buds had the best proliferation effect and the biomass gain multiple was 13.52. Secondly, when 0.2 mg·L<sup>-1</sup> IBA was added, the biomass gain multiple reached 11.60. When the IBA concentration was 0 mg·L<sup>-1</sup>, the biomass gain multiple of advent buds was the lowest. Therefore, when 0.3 mg·L<sup>-1</sup> IBA was added, the adventitious bud growth of *O. triangularis* 'Purpurea' was the best.

### Effects of different concentrations of IBA and NAA on adventitious buds rooting and bulb induction

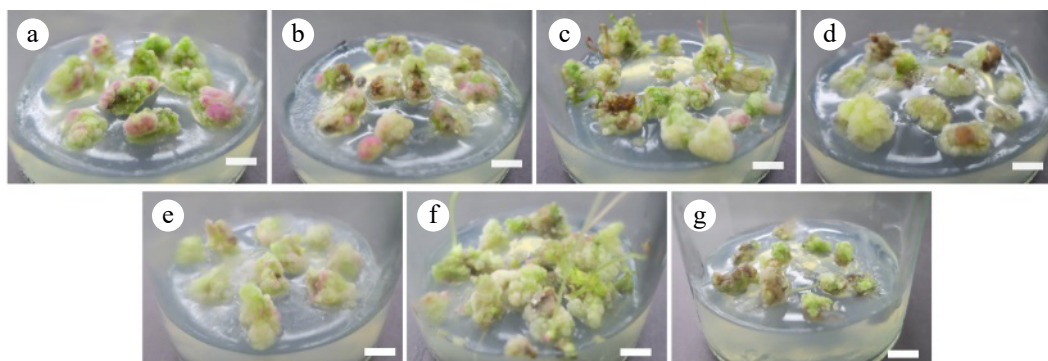
Without NAA, the overall rooting number was less than when NAA was supplemented, and the growth state of the plants was worse. When the NAA concentration was 0.3 mg·L<sup>-1</sup>, with the increase in IBA concentration, the average number of roots, average root length, and average height showed an increasing trend. When NAA concentration was 0.5 mg·L<sup>-1</sup>, there was no significant difference in average height, and the growth state was better. The average number of roots generally increased and then decreased, while the average root length showed a trend of gradual decrease (Table 5). However, when the NAA was not added and whether IBA was added or not, the rooting effect was poor and the plant growth was bad. In the treatment group of 0.3 mg·L<sup>-1</sup> NAA and 0.3 mg·L<sup>-1</sup> IBA, the rooting effect was the best. The rooting rate was 100%. The average number of roots was 28.16 per plant. The average root length was 1.96 cm and the average height was 6.62 cm.

In the process of inducing rooting, small bulbs grew at the base of the plant. When NAA was not added and the IBA concentration was 0, 0.1, 0.3 mg·L<sup>-1</sup>, the bulb induction effect was poor (Fig. 5a–c). When the NAA concentration was 0.3 mg·L<sup>-1</sup>, the number of small bulbs induced by IBA supplemented medium was significantly higher than that without IBA, and the growth state of bulbs was better (Fig. 5d–f). When

**Table 3.** Effects of different 6-BA and NAA concentrations on adventitious bud rooting.

Treatment	6-BA concentration (mg·L <sup>-1</sup> )	NAA concentration (mg·L <sup>-1</sup> )	Biomass gain multiple	Callus multiplier multiple	Callus texture and color
D1	0.50	0.20	3.23 ± 0.14b	3.22 ± 0.18a	Purple, loose
D2	1.50	0.20	4.23 ± 0.16a	3.47 ± 0.12a	White, green, few bud points
D3	2.50	0.20	3.37 ± 0.16b	3.29 ± 0.15a	White, green, few bud points of granular callus
D4	0.50	0.40	3.54 ± 0.67b	3.19 ± 0.10a	White
D5	1.50	0.40	3.43 ± 0.14b	3.33 ± 0.08a	White, dense partial bud points
D6	2.50	0.40	4.05 ± 0.37a	3.39 ± 0.29a	Green, multiple differentiation numbers
CK	0.00	0.00	1.93 ± 0.09c	1.86 ± 0.07b	Green, browning on the lower part, low proliferation numbers

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ( $p < 0.05$ ).



**Fig. 4** Callus proliferation effect. (a)–(g) Correspond to the results of callus-induced callus proliferation in D1–D6 and CK groups for 30 d. Scale bar = 1 cm.

**Table 4.** Effects of different IBA concentrations on the induction of adventitious buds.

Treatment	Concentrations of IBA (mg·L <sup>-1</sup> )	Biomass gain multiple	Plant status
CK	0.00	4.51 ± 0.94e	Slender petioles
C1	0.10	6.92 ± 0.46c	Slender and long petioles green leaves
C2	0.20	11.60 ± 0.40b	Purple petioles big leaves
C3	0.30	13.52 ± 0.41a	Green petioles and leaves granular calli
C4	0.40	5.95 ± 0.50cd	Short plants
C5	0.50	5.88 ± 0.41cd	Short plants

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ( $p < 0.05$ ).

the NAA and IBA concentration was 0.5 mg·L<sup>-1</sup> and 0.00–0.30 mg·L<sup>-1</sup> respectively, there was no significant difference in the number of small bulbs induced, and it had a good induction effect (Fig. 5g–i).

### Sterile bulb transplanting

Bulbs were removed from sterile bottles (Fig. 6a). The germination rate was 34.31% at 10 d after transplantation. At 20 d, the germination rate was 57.84% and the bulbs grew into healthy plants at 30 d (Fig. 6b). At 45 d of transplanting, the plants could form flower buds and flowers, and the survival rate was up to 100% (Fig. 6c).

## Discussion

### Leaves and petioles were selected as explants to induce calli

The selection of appropriate explants is crucial for inducing embryonic callus. The effect of different explants on callus induction is different<sup>[18]</sup>. High-quality calli are beneficial to the

formation of adventitious buds and the improvement of plant tissue culture efficiency<sup>[19]</sup>. In the callus induction of *O. triangularis* 'Purpurea', petioles and leaves are the most common explants. In a few cases, bulbs are used as explants for callus induction. Researchers believed that leaves were the best explants for inducing callus and adventitious bud differentiation<sup>[20]</sup>. Tang et al.<sup>[21]</sup> discovered that adventitious buds were induced easily when scales were used as explants of *Lilium leucanthum*. In the appropriate medium, more calli were induced when induction was carried out with scales. However, compared with *L. leucanthum*, the callus of *O. triangularis* 'Purpurea' is difficult to induce. When the basal plate was used as explants to induce callus, the callus formation time was longer and the volume was less. Therefore, bulbs and basal plates are not suitable for callus induction. This study selected leaves and petioles of *O. triangularis* 'Purpurea' as two different explants. The quality of calli induced by them had significant differences. As explants, petioles had the best effect on callus induction. It was consistent with the research results of Suzana et al.<sup>[22]</sup>.

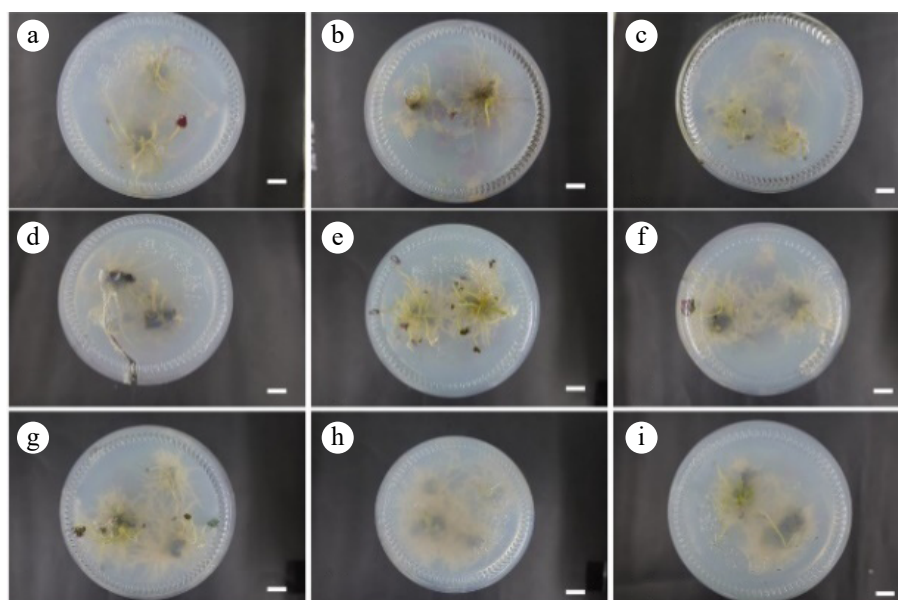
### Exogenous plant growth regulators affect embryogenic callus induction

Exogenous plant growth regulators are one of the important factors in inducing embryogenic callus<sup>[5]</sup>. In the study of callus induction, commonly used auxins are NAA, IBA, and 2,4-D<sup>[23,24]</sup>. The main cytokinins are 6-BA<sup>[25]</sup> and KT<sup>[26]</sup>. Rittirat et al.<sup>[27]</sup> found that when explants were the petioles of *O. triangularis* 'Purpurea', adding suitable 2,4-D was conducive to callus induction and the acceleration of adventitious buds growth. Xu et al.<sup>[26]</sup> found that the combination of 0.5 mg·L<sup>-1</sup> KT and a certain concentration of 2,4-D was beneficial to callus induction in *Hedera nepalensis* var. *sinensis*. Few scholars have used TDZ to induce the callus of *O. triangularis* 'Purpurea'. TDZ is a synthetic plant growth regulator. It has a significant effect on callus induction and differentiation<sup>[28]</sup>. A low concentration of TDZ

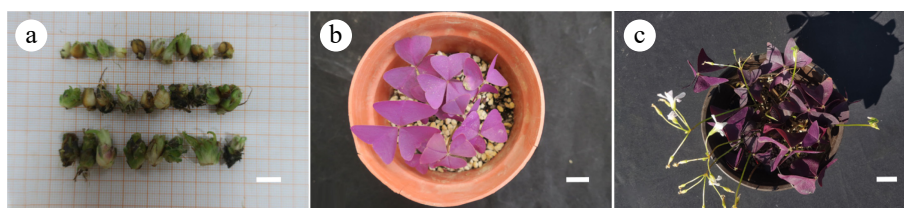
**Table 5.** Effects of different concentrations of IBA and NAA on rooting.

Treatment	Concentrations of IBA (mg·L <sup>-1</sup> )	Concentrations of NAA (mg·L <sup>-1</sup> )	Rooting rate (%)	Average number of roots (bar·plant <sup>-1</sup> )	Average root length (cm)	Height (cm)	Average bulb quantity (pcs)	Root growth status
R1	0.00	0.00	91.11 ± 2.48f	7.11 ± 0.08f	1.82 ± 0.15abc	5.71 ± 0.31bcd	0.51 ± 0.32cd	Lateral roots on the taproot, large leaves
R2	0.10	0.00	92.22 ± 3.06e	5.33 ± 0.49g	1.53 ± 0.41abcde	5.10 ± 0.46d	0.95 ± 0.93bcd	Few roots, long taproots
R3	0.30	0.00	91.11 ± 2.48f	7.05 ± 0.91f	1.43 ± 0.25bcde	6.13 ± 0.10abc	0.00 ± 0.00d	Few roots, thick taproots, no lateral root
R4	0.00	0.30	93.33 ± 2.36d	6.33 ± 0.49fg	1.18 ± 0.10de	5.52 ± 0.63cd	0.37 ± 0.19d	Thick taproots, no lateral root
R5	0.10	0.30	91.11 ± 2.48f	12.89 ± 0.87d	1.63 ± 0.12abcd	6.46 ± 0.16ab	1.99 ± 0.79a	Very thick taproots, no lateral root
R6	0.30	0.30	100 ± 0.0a	28.16 ± 0.51a	1.96 ± 0.16a	6.62 ± 0.55ab	2.12 ± 0.44a	Thick roots, no lateral roots, numerous roots
R7	0.00	0.50	95.56 ± 3.42b	26.08 ± 0.33b	1.88 ± 0.06ab	7.04 ± 0.30a	2.24 ± 0.30a	Thick taproots, no lateral root
R8	0.10	0.50	94.44 ± 5.48c	28.25 ± 0.80a	1.40 ± 0.11cde	6.63 ± 0.18ab	1.50 ± 0.84abc	Numerous and dense roots, but short, no lateral root
R9	0.30	0.50	95.56 ± 6.34b	19.85 ± 0.40c	1.15 ± 0.14e	6.84 ± 0.37a	1.85 ± 0.46ab	Thin, short, numerous root

The data are mean ± standard deviation, and different letters after the same column of numbers indicate significant differences between different treatments ( $p < 0.05$ ).



**Fig. 5** Rooting results at 30 d. (a)–(i) Correspond to the 30 d rooting results of the R1–R9 groups, respectively. Scale bar = 1 cm.



**Fig. 6** Transplanting of sterile bulbs. (a) Sterile bulbs of different sizes. (b) Growth state of sterile bulbs transplanted for 20 d. (c) Growth state of sterile bulbs transplanted for 45 d. Scale bar = 1 cm.

can promote cell proliferation significantly<sup>[29]</sup>. The studies of *Acer rubrum*<sup>[14]</sup>, *Sophora davidii*<sup>[15]</sup>, and *Lens culinaris*<sup>[30]</sup> discovered that TDZ was beneficial to the induction of embryonic callus. In this study, the petioles and leaves of *O. triangularis* 'Purpurea' were used as explants for induction. In the medium supplemented with NAA and 6-BA, the granular callus induction rate was 15.43%. In the TDZ-supplemented medium, petioles induced white loose callus within 12 d. At 30 d, up to 53.33% of petioles formed close-arranged round granular callus. These results indicated that an appropriate concentration of TDZ could significantly promote the induction rate and callus quality of embryonic callus of *O. triangularis* 'Purpurea'. When the petioles were placed on a medium with high TDZ concentration ( $2.50 \text{ mg}\cdot\text{L}^{-1}$ ) for 30–45 d, the callus induction effect was good and the growth rate was fast in a short time. However, after 60 d of inoculation, calli showed growth retardation and browning. If embryogenic callus status is to be maintained, the TDZ should be evacuated in time. To maintain embryogenic callus status, calli were inoculated on MS medium containing 6-BA and NAA. When the ratio of 6-BA to NAA was 6:1 to 10:1, the callus multiplier rate was the highest, and the multiplication ratio was 3.39 or 3.47 at 30 d. Murthy et al.<sup>[31]</sup> believed that the growth retardation of callus may result from disrupting the optimal balance of plant hormones in the tissue, or the high concentration of TDZ that increased stress on the plant. Long-term inoculation with a medium containing high auxin would result in somatic embryo deformities and even make calli lose embryogenesis gradually. Therefore, many researchers reduced the concentration of plant growth regulators to a suitable level

for the proliferation or maintenance of embryogenic callus status. However, because plant cells at different developmental stages have different sensitivity to hormones, the concentration of growth regulators should be constantly adjusted to maintain embryogenic callus status during the process of proliferation.

### Morphology of embryogenic calli in different periods

Embryogenic calli grew faster than non-embryogenic calli, and plants were regenerated through the somatic embryogenic pathway<sup>[32]</sup>. In this experiment, the white villous callus I did not belong to embryonic callus, and the growth rate of callus was slow in this state. This was contrary to the result that the white callus induced in the subculture of *Picea mongolica* was an embryonic callus<sup>[33]</sup>. The reason may be that the situation of callus induction was slightly different among different species. Over time, the white callus gradually changed into the loose callus of callus II and the granular callus of callus III. This was consistent with the result that initially induced calli of *Triticum aestivum* were transformed into embryogenic calli after several subculture times<sup>[34]</sup>.

### Conclusions

In conclusion, this study established the regeneration system of leaves and petioles of *Oxalis triangularis* 'Purpurea'. The medium of petiole callus induction was MS +  $1.00 \text{ mg}\cdot\text{L}^{-1}$  6-BA +  $0.50 \text{ mg}\cdot\text{L}^{-1}$  NAA +  $1.00 \text{ mg}\cdot\text{L}^{-1}$  TDZ. Granular calli have a

strong ability to divide and regenerate. In this medium, the granular callus induction rate was the highest. The medium for petiole callus differentiation and leaf callus induction and differentiation were MS + 1.00 mg·L<sup>-1</sup> 6-BA + 0.50 mg·L<sup>-1</sup> NAA. Callus proliferation medium was MS + 1.50 mg·L<sup>-1</sup> 6-BA + 0.20 mg·L<sup>-1</sup> NAA. The callus-induced adventitious buds were inoculated onto 1/2 MS + 0.30 mg·L<sup>-1</sup> NAA + 0.30 mg·L<sup>-1</sup> IBA rooting medium. The rooting rate reached 100% at 30 d. Around 75–90 d, a large number of small bulbs could be induced, and complete plants can be formed. This establishment of an *in vitro* regeneration system provides useful reference and experimental basis for factory production, genetic engineering and genetic transformation of *O. triangularis* 'Purpurea'.

## Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Xiao Y, Wu S; data collection: Chen S, Xiao Y, Wang X, Wu K, Shen S; analysis and interpretation of results: Chen S, Xiao Y; draft manuscript preparation: Chen S, Zhai J, Wu S; supervision: Wu S. All authors reviewed the results and approved the final version of the manuscript.

## Data availability

All data generated or analyzed during this study are included in this published article.

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## Conflict of interest

The authors declare that they have no conflict of interest. Shasha Wu is the Editorial Board member of *Technology in Horticulture* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and the research groups.

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