Different Amino Acids Inhibit or Promote Rhizome Proliferation and Differentiation in *Cymbidium goeringii*

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Abstract. The proliferation and differentiation of rhizomes are crucial for the propagation of *Cymbidium* species. We systematically assessed the effects of different concentrations of 20 amino acids on the proliferation and differentiation of *C. goeringii* rhizomes. Rhizome proliferation rates were significantly higher in media with 2.0 mmol·L⁻¹ cysteine, 0.5 mmol·L⁻¹ arginine, 0.2 mmol·L⁻¹ asparagine, 1.0 mmol·L⁻¹ proline, and 0.5 mmol·L⁻¹ lysine compared with those in the control. Additionally, 1.0 mmol·L⁻¹ tyrosine, 0.5 mmol·L⁻¹ asparagine, and 0.2 mmol·L⁻¹ aspartate were beneficial for rhizome differentiation. Furthermore, two combinations of amino acids, 0.5 mmol·L⁻¹ arginine + 1.0 mmol·L⁻¹ proline and 0.5 mmol·L⁻¹ arginine + 2.0 mmol·L⁻¹ cysteine, resulted in proliferation rates of 3.05 and 3.01, respectively, after 60 days. The highest differentiation rate (5.39 after 60 days) was observed in media with 0.5 mmol·L⁻¹ asparagine + 0.2 mmol·L⁻¹ aspartate. This study demonstrated that certain combinations of amino acids can effectively promote the proliferation and differentiation of rhizomes during the rapid propagation of *C. goeringii*.

Cymbidium goeringii is a famous ornamental flower in China and other Asian countries that has commercially valued characteristics. Recently, studies of *C. goeringii* have focused mainly on the molecular mechanisms controlling flowering time (Huang et al., 2012; Ren et al., 2020a; Yang et al., 2019; Zhang et al., 2019), floral scent (Ramya et al., 2019), floral organ development (Xiang et al., 2018; Yang et al., 2017), and stress physiology (Ren et al., 2020b). However, this popular species is endangered due to illegal collection and habitat destruction (Chung and Nason, 2007). Currently, the most common propagation method of *C. goeringii* is through the separation of pseudobulbs; however, this is too inefficient for commercial production. Tissue culture is a rapid and efficient way to propagate *C. goeringii*. During propagation of *C. goeringii*, mature seeds are often used as the material to obtain sterile rhizomes for further proliferation and differentiation. The rhizome stage is advantageous for the propagation of *C. goeringii* (Roy and Banerjee, 2002). However, the proliferation

and differentiation rates are slow, and some rhizomes can turn brown, which limits further growth (Paek and Kozai, 1998). Therefore, the optimization of rhizome proliferation and differentiation under culture conditions can promote the rapid propagation of *C. goeringii*.

Recently, some progress has been made in the study of rhizome proliferation and differentiation of *Cymbidium*. Activated charcoal (AC) can effectively promote *C. sinense* rhizome proliferation and prevent browning (Gao et al., 2014; Lee et al., 2011). Additionally, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) can effectively induce rhizome proliferation in *Cymbidium* (Park et al., 2018; Shimasaki and Uemoto, 1990). Importantly, the combination of kiwifruit juice and sodium citrate can effectively promote rhizome proliferation and prevent browning of the rhizome during the micropropagation of *C. goeringii* (Huang et al., 2017). In terms of rhizome differentiation, supplementing 6-benzylaminopurine (6-BA) and NAA to the culture medium can promote shoot induction from the rhizome. Park et al. (2018) reported that medium fortified with 20 μM 2,4-D, and 2 μM thidiazuron was most effective for inducing adventitious buds. Cutting the rhizome explants into 1-cm segments resulted in massive shoot formation (Gao et al., 2014). Moreover, a recent study revealed that YUC-mediated auxin biogenesis is involved in shoot regeneration from the rhizome in *Cymbidium* (Liu et al., 2017).

The rapid propagation technology used for *C. goeringii* (Park et al., 2018) and other

Cymbidium species (Gao et al., 2014; Guha and Rao, 2012; Lee et al., 2011; Liu et al., 2017) has received considerable attention over the years, but little work has focused specifically on the effects of amino acids in tissue culture. As fundamental nutrients, amino acids can be synthesized into many important compounds (such as plant hormones and secondary metabolites) (Krapp, 2015; Tegeder, 2012). Amino acids not only have an important role in the plant nitrogen balance but also control plant growth and development, flowering, and seed yield (Coruzzi and Bush, 2001). Experiments confirmed that amino acid alanine could promote plant growth (Ichihashi et al., 2020). Glutamate can promote rice growth at low concentrations and inhibit rice growth at high concentrations (Kan et al., 2017). Additionally, certain concentrations of amino acids, such as lysine and arginine, can promote the growth of rice plants and tiller bud elongation (Lu et al., 2018; Wang et al., 2019).

It is unknown whether amino acids can promote the growth of *C. goeringii* rhizomes. This study demonstrated that certain combinations of amino acids can effectively promote the proliferation and differentiation of rhizomes in the rapid propagation of *C. goeringii* via tissue culture. These findings could provide a theoretical basis and technical support for improving the propagation speed of *Cymbidium*.

Materials and Methods

In vitro seed germination and rhizome development. Mature pods (150 d after pollination) of *C. goeringii* were harvested from a greenhouse at Guizhou University in Guiyang, China. The pods were surface-sterilized by dipping in 70% ethanol for 30 s, immersing in 0.1% (w/v) HgCl₂ solution for 10 min, and rinsing five times with sterile distilled water. Then, the pods were cut with a sterile blade, and the seeds were scraped out with the blade for the next experiments. The seed germination capacity was investigated on Murashige and Skoog (MS) media containing 0.5 mg·L⁻¹ NAA, 30 g·L⁻¹ sucrose, 2 g·L⁻¹ AC, and 100 mL·L⁻¹ coconut milk. The coconut milk was obtained from 6- to 7-month-old green coconuts and filtered through filter paper. Seed germination and rhizome development were investigated after 90 d.

In vitro proliferation of rhizomes. Rhizomes from immature seed were rinsed twice in sterilized water to remove the culture media. Then, 5-mm-long rhizome apical segments were placed on MS media containing 0.2, 0.5, 1.0, or 2.0 mmol·L⁻¹ of nonpolar amino acids (glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, or methionine), polar neutral amino acids (serine, threonine, glutamine, tyrosine, asparagine, or cysteine), polar acidic amino acids (glutamate or aspartate), or polar basic amino acids (histidine, arginine, or lysine). Media were also supplemented with 5 mg·L⁻¹ NAA, 1.5 mg·L⁻¹ 6-BA, 2 g·L⁻¹ AC, and 30

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g·L⁻¹ sucrose. Medium without amino acids served as a control. After determining the effects of individual amino acids on the proliferation of rhizomes, different combinations of the amino acids that promoted proliferation were examined using the same methods. There were three replicates of each treatment, with 200 rhizomes per replicate. The rhizome proliferation rate was calculated as the rhizome fresh weight after 60 d of culturing compared with the rhizomes before culture.

Shoot differentiation from rhizomes. The effects of amino acids on shoot differentiation from rhizomes were investigated using the same methods and treatments for amino acids that were used in rhizome proliferation, except 10-mm rhizome apical segments were used. In addition to amino acids, media were supplemented with 5 mg·L⁻¹ 6-BA, 0.2 mg·L⁻¹ NAA, and 20 g·L⁻¹ sucrose. Medium without amino acids served as a control. The rhizome differentiation rate was calculated as the average number of shoots per rhizome after 60 d of culturing.

Culture conditions and statistical analysis. The media were adjusted to pH 5.4 before autoclaving at 121 °C for 20 min at 1.06 kg·cm⁻². All cultures were incubated in a controlled environment growth room at 25 ± 2 °C with a 16-h photoperiod under cool white light (40–50 μmol·m⁻²·s⁻¹). All experiments were set according to a completely random design. The data were analyzed using one-way analyses of variance and Duncan's multiple range tests at the 5% level.

Results

Effects of nonpolar amino acids on *C. goeringii* rhizome proliferation and differentiation. Proline at 1.0 mmol·L⁻¹ and isoleucine at 0.2 mmol·L⁻¹ significantly increased the proliferation of *C. goeringii* rhizomes compared with the control (Fig. 1A and B). Proliferation rates reached 2.24 and 1.81 under these two treatments, respectively. Although some concentrations of glycine, alanine, valine, and tryptophan resulted in higher proliferation rates compared with the control, the differences were not statistically significant (Table 1). Under these treatments, rhizomes grew well and were light green. Phenylalanine and methionine resulted in proliferation rates similar to those of the control (Table 1). Higher concentrations of leucine inhibited rhizome growth (Table 1). Furthermore, high concentrations of isoleucine and all concentrations of phenylalanine resulted in browning of rhizomes during proliferation (Table 1).

Valine at 0.5 mmol·L⁻¹, leucine at 0.5 mmol·L⁻¹, isoleucine at 0.2 mmol·L⁻¹, tryptophan at 0.2 mmol·L⁻¹, and tryptophan at 1.0 mmol·L⁻¹ had no effect on the differentiation of rhizomes compared with the control (Fig. 1A). All concentrations of glycine, alanine, proline, phenylalanine, and methionine inhibited rhizome differentiation (Table 1). The addition of nonpolar amino acids caused widespread browning during

Fig. 1. Effects of different amino acid treatments on *Cymbidium goeringii* rhizome proliferation. (A) Control. (B) Proline at 1.0 mmol·L⁻¹. (C) Cysteine at 2.0 mmol·L⁻¹. (D) Asparagine at 0.2 mmol·L⁻¹. (E) Aspartate at 2.0 mmol·L⁻¹. (F) Arginine at 0.5 mmol·L⁻¹. (G) Lysine at 0.5 mmol·L⁻¹. (H) Arginine at 0.5 mmol·L⁻¹ + cysteine at 2.0 mmol·L⁻¹. (I) Arginine at 0.5 mmol·L⁻¹ + proline at 1.0 mmol·L⁻¹.

differentiation. The highest level of inhibition occurred under methionine at 2.0 mmol·L⁻¹, but no browning occurred in this treatment (Fig. 2B). In summary, no nonpolar amino acids promoted the differentiation of rhizomes.

Effects of polar neutral amino acids on *C. goeringii* rhizome proliferation and differentiation. As shown in Table 2 and Fig. 1, cysteine at 2.0 mmol·L⁻¹ (Fig. 1C) and asparagine at 0.2 mmol·L⁻¹ (Fig. 1D) significantly increased rhizome proliferation rates of *C. goeringii* compared with the control, with proliferation rates of 2.35 and 2.33, respectively. Higher concentrations of asparagine inhibited rhizome growth (Table 2). Cysteine at 0.5 and 1.0 mmol·L⁻¹ also resulted in significantly higher rhizome proliferation compared with the control (Table 2). Different concentrations of serine had no effect on rhizome proliferation (Table 2). However, threonine 0.2 mmol·L⁻¹ and tyrosine 1.0 mmol·L⁻¹ significantly promoted the proliferation of rhizomes compared with the control (Table 2). Glutamine was not beneficial to rhizome proliferation (Table 2). Rhizome browning was not observed under any polar neutral amino acid treatments.

The differentiation of rhizomes was significantly promoted under asparagine at 0.5 mmol·L⁻¹ (Fig. 2C), which resulted in four buds per rhizome (Table 2). Tyrosine (at 0.2, 0.5, 1.0, or 2.0 mmol·L⁻¹), asparagine (at 1.0 or 2.0 mmol·L⁻¹), and cysteine (at 0.5 mmol·L⁻¹) also significantly promoted rhizome differentiation compared with the control, resulting in more than three buds per

rhizome (Table 2). Different concentrations of serine and threonine significantly inhibited the differentiation of rhizome compared with the control (Table 2). Glutamine had no effect on rhizome differentiation compared with the control (Table 2). All concentrations of serine and threonine significantly inhibited rhizome differentiation compared with the control, resulting in fewer than two buds per rhizome (Table 2). The addition of nonpolar amino acids caused browning during differentiation, but not during proliferation.

Effects of polar acidic amino acids on *C. goeringii* rhizome proliferation and differentiation. Low concentrations of glutamate (0.2 mmol·L⁻¹ and 0.5 mmol·L⁻¹) significantly promoted rhizome proliferation of *C. goeringii* compared with the control (Table 3). The proliferation rate was 1.82 when glutamate was added at 0.2 mmol·L⁻¹. Under this treatment, rhizome growth was normal and the rhizome was green. The proliferation rate decreased under increasing glutamate concentrations, with inhibition under 1.0 mmol·L⁻¹ and increasing inhibition under higher concentrations (Table 3). Aspartate did not increase rhizome proliferation, and higher concentrations of aspartate inhibited rhizome growth and resulted in rhizome browning (Fig. 1E).

Low concentrations of aspartate (0.2 mmol·L⁻¹) significantly increased rhizome differentiation compared with the control, resulting in more than four buds per rhizome (Table 3). Glutamate resulted in lower rhizome differentiation rates compared with the control (Table 3). Both polar acidic amino

Table 1. Effects of different nonpolar amino acid treatments on *C. goeringii* rhizome proliferation and differentiation.

Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation	Differentiation rate	Browning status during differentiation
CK	1.35 ± 0.13 defghijk	Slight	2.73 ± 0.17 a	Slight
Glycine 0.2	1.01 ± 0.17 jkl	No	2.07 ± 0.10 bcd	Moderate
Glycine 0.5	1.67 ± 0.38 bcde	No	1.43 ± 0.09 fghi	Slight
Glycine 1.0	1.43 ± 0.20 bcdefgh	No	1.44 ± 0.09 fghi	Moderate
Glycine 2.0	1.36 ± 0.36 defghijkl	No	1.41 ± 0.15 fghi	Moderate
Alanine 0.2	1.48 ± 0.45 bcdefgh	No	1.88 ± 0.16 cdef	No
Alanine 0.5	1.65 ± 0.52 bcde	No	1.98 ± 0.15 bcd	Moderate
Alanine 1.0	1.32 ± 0.13 defghijkl	No	2.18 ± 0.15 bcd	Moderate
Alanine 2.0	1.19 ± 0.04 hijkl	No	1.92 ± 0.17 bcd	Moderate
Valine 0.2	1.23 ± 0.14 hijkl	No	2.16 ± 0.15 bcd	Slight
Valine 0.5	1.32 ± 0.10 defghijkl	No	2.41 ± 0.13 ab	Slight
Valine 1.0	1.64 ± 0.09 bcdef	No	1.87 ± 0.16 cdef	Slight
Valine 2.0	1.47 ± 0.07 bcdefghi	No	1.77 ± 0.12 defg	Slight
Leucine 0.2	1.37 ± 0.30 defghijk	No	2.22 ± 0.12 bc	Moderate
Leucine 0.5	1.53 ± 0.35 bcdefgh	No	2.80 ± 0.24 a	Slight
Leucine 1.0	1.14 ± 0.13 ijkl	No	1.72 ± 0.16 defg	Slight
Leucine 2.0	1.09 ± 0.69 jkl	No	1.69 ± 0.18 defg	Slight
Isoleucine 0.2	1.81 ± 0.11 b	No	3.05 ± 0.20 a	Moderate
Isoleucine 0.5	1.35 ± 0.18 defghijk	No	2.28 ± 0.11 bc	Slight
Isoleucine 1.0	1.18 ± 0.31 hijkl	Slight	2.24 ± 0.15 bc	Slight
Isoleucine 2.0	1.15 ± 0.24 hijkl	Slight	2.20 ± 0.14 bc	Slight
Proline 0.2	1.43 ± 0.16 cdefghij	Severe	2.23 ± 0.15 bc	Slight
Proline 0.5	1.54 ± 0.24 bcdefgh	Slight	2.12 ± 0.16 bcd	Slight
Proline 1.0	2.24 ± 0.54 a	No	1.73 ± 0.13 defg	Slight
Proline 2.0	1.52 ± 0.23 bcdefgh	No	1.69 ± 0.15 defg	Slight
Phenylalanine 0.2	1.26 ± 0.08 ghijk	Severe	1.73 ± 0.08 defg	Moderate
Phenylalanine 0.5	1.31 ± 0.21 efghijkl	Severe	1.51 ± 0.14 efghi	Severe
Phenylalanine 1.0	1.38 ± 0.19 defghijk	Severe	2.0 ± 0.15 bcd	Moderate
Phenylalanine 2.0	1.15 ± 0.08 ijkl	Severe	1.65 ± 0.14 efg	Moderate
Tryptophan 0.2	1.24 ± 0.25 ghijk	Slight	2.94 ± 0.11 a	Severe
Tryptophan 0.5	1.35 ± 0.26 defghijk	Slight	2.01 ± 0.10 bcd	Moderate
Tryptophan 1.0	1.64 ± 0.18 bcdef	No	2.5 ± 0.12 ab	Moderate
Tryptophan 2.0	1.50 ± 0.37 bcdefgh	No	2.12 ± 0.15 bcd	Moderate
Methionine 0.2	0.97 ± 0.23 jkl	Slight	2.18 ± 0.15 bcd	Moderate
Methionine 0.5	1.28 ± 0.38 ghijk	Slight	1.19 ± 0.10 hij	No
Methionine 1.0	1.19 ± 0.22 ijkl	Slight	0.86 ± 0.08 ij	No
Methionine 2.0	1.14 ± 0.22 ijkl	Slight	0.81 ± 0.12 ij	No

Fig. 2. Effects of different amino acid treatments on *Cymbidium goeringii* rhizome differentiation. (A) Control. (B) Methionine at 2.0 mmol·L⁻¹. (C) Asparagine at 0.5 mmol·L⁻¹. (D) Asparagine at 0.5 mmol·L⁻¹ + tyrosine at 1.0 mmol·L⁻¹. (E) Asparagine at 0.5 mmol·L⁻¹ + aspartate at 0.2 mmol·L⁻¹. (F) Shoots developed from the tips of the rhizomes in Huabao medium supplemented with activated charcoal (AC) and naphthalene acetic acid (NAA) after 2 months.

acids caused severe browning during rhizome differentiation.

Effects of polar basic amino acids on *C. goeringii* rhizome proliferation and differentiation. Rhizome proliferation under histidine treatments did not differ significantly from that under the control, except for at 2.0 mmol·L⁻¹, when proliferation was significantly lower compared with that under the control of *C. goeringii* (Table 4). The addition of arginine at

0.5 mmol·L⁻¹ significantly increased proliferation rates compared with the control, resulting in a proliferation rate of 2.32 (Fig. 1F). Proliferation rates decreased with increasing arginine concentrations, and rhizome browning occurred. Lysine at 0.5 mmol·L⁻¹ also resulted in higher rhizome proliferation compared with the control, with a proliferation rate of 2.21 (Fig. 1G). No rhizome browning occurred under lysine treatments.

All concentrations of histidine and lysine significantly inhibited rhizome differentiation (Table 4). Low concentrations (0.2 mmol·L⁻¹) of arginine had no significant effect on rhizome differentiation compared with the control; however, with increasing arginine concentrations, the inhibition of rhizome differentiation became obvious (Table 4). All polar basic amino acids except arginine at 0.5 mmol·L⁻¹ caused widespread browning during differentiation.

Effects of amino acid combinations on *C. goeringii* rhizome proliferation and differentiation. Arginine at 0.5 mmol·L⁻¹ + proline at 1.0 mmol·L⁻¹ (Fig. 1I) and arginine at 0.5 mmol·L⁻¹ + cysteine at 2.0 mmol·L⁻¹ (Fig. 1H) obviously promoted the growth of rhizomes more than other treatments, resulting in proliferation rates of 3.05 and 3.01, respectively. Other combinations also promoted the proliferation of rhizomes, and browning was only observed under the amino acid combinations of cysteine + threonine and cysteine + glutamate (Table 5).

Asparagine at 0.5 mmol·L⁻¹ + tyrosine at 1.0 mmol·L⁻¹ (Fig. 2D) and asparagine at 0.5 mmol·L⁻¹ + aspartate at 0.2 mmol·L⁻¹ (Fig. 2E) resulted in significantly higher rhizome differentiation rates compared with the other combinations tested, with differentiation rates of 5.34 and 5.39, respectively (Table 6). All the combinations of amino acids caused a certain degree of browning.

Table 2. Effects of different polar neutral amino acid treatments on *C. goeringii* rhizome proliferation and differentiation.

Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation	Differentiation rate	Browning status during differentiation
Control	1.36 ± 0.13 defgh	Slight	2.73 ± 0.17 c	Slight
Serine 0.2	1.26 ± 0.21 efgh	No	1.8 ± 0.08 efg	Moderate
Serine 0.5	1.63 ± 0.29 bcde	No	1.79 ± 0.10 efg	Moderate
Serine 1.0	1.33 ± 0.24 defgh	No	1.84 ± 0.09 efg	Moderate
Serine 2.0	1.18 ± 0.09 fgh	No	1.75 ± 0.12 efg	Moderate
Threonine 0.2	1.86 ± 0.33 bc	No	1.6 ± 0.17 fg	Severe
Threonine 0.5	1.75 ± 0.34 bcd	No	1.74 ± 0.14 efg	Severe
Threonine 1.0	1.69 ± 0.33 bcd	No	1.29 ± 0.08 gh	Moderate
Threonine 2.0	1.57 ± 0.28 bcdef	No	1.22 ± 0.12 gh	Moderate
Glutamine 0.2	1.35 ± 0.40 cdefgh	No	2.18 ± 0.19 cd	Slight
Glutamine 0.5	1.38 ± 0.43 defgh	No	2.79 ± 0.17 c	Slight
Glutamine 1.0	1.08 ± 0.11 h	No	2.74 ± 0.22 c	Moderate
Glutamine 2.0	1.12 ± 0.14 gh	No	2.69 ± 0.18 c	Moderate
Tyrosine 0.2	1.55 ± 0.53 bcdef	No	3.12 ± 0.23 b	Slight
Tyrosine 0.5	1.72 ± 0.45 bcd	No	3.02 ± 0.18 b	Moderate
Tyrosine 1.0	1.81 ± 0.32 b	No	4.34 ± 0.15 ab	Severe
Tyrosine 2.0	1.55 ± 0.42 bcdefg	No	3.79 ± 0.18 b	Severe
Asparagine 0.2	2.33 ± 0.14 a	No	2.96 ± 0.14 c	Moderate
Asparagine 0.5	1.84 ± 0.06 b	No	4.66 ± 0.24 a	Severe
Asparagine 1.0	1.62 ± 0.05 bcde	No	4.44 ± 0.24 ab	Moderate
Asparagine 2.0	1.52 ± 0.15 bcdefg	No	3.89 ± 0.18 b	Moderate
Cysteine 0.2	1.67 ± 0.36 bcd	No	2.76 ± 0.26 c	Moderate
Cysteine 0.5	1.84 ± 0.39 b	No	3.24 ± 0.35 b	Slight
Cysteine 1.0	1.90 ± 0.33 b	No	2.67 ± 0.31 c	Slight
Cysteine 2.0	2.35 ± 0.16 a	No	2.11 ± 0.27 cd	Slight

Table 3. Effects of different polar acidic amino acid treatments on *C. goeringii* rhizome proliferation and differentiation.

Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation	Differentiation rate	Browning status during differentiation
CK	1.36 ± 0.13 bc	Slight	2.73 ± 0.17 b	Slight
Glutamate 0.2	1.82 ± 0.38 a	No	2.26 ± 0.11 c	Severe
Glutamate 0.5	1.57 ± 0.22 ab	No	2.33 ± 0.11 bc	Severe
Glutamate 1.0	1.31 ± 0.05 bc	No	2.25 ± 0.12 c	Severe
Glutamate 2.0	1.06 ± 0.08 c	No	2.15 ± 0.12 c	Severe
Aspartate 0.2	1.38 ± 0.19 bc	No	4.19 ± 0.17 a	Severe
Aspartate 0.5	1.35 ± 0.21 bc	No	2.62 ± 0.17 bc	Moderate
Aspartate 1.0	1.62 ± 0.24 b	No	2.48 ± 0.13 bc	Severe
Aspartate 2.0	0.88 ± 0.10 d	Severe	2.24 ± 0.12 c	Severe

Table 4. Effects of different polar basic amino acid treatments on *C. goeringii* rhizome proliferation and differentiation.

Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation	Differentiation rate	Browning status during differentiation
CK	1.36 ± 0.13 efg	Slight	2.733 ± 0.17 a	Slight
Histidine 0.2	1.15 ± 0.11 fgh	No	2.28 ± 0.11 b	Moderate
Histidine 0.5	1.19 ± 0.22 gh	No	1.91 ± 0.09 bcd	Slight
Histidine 1.0	1.15 ± 0.28 gh	No	2.14 ± 0.11 bc	Moderate
Histidine 2.0	1.03 ± 0.16 h	No	2.01 ± 0.12 bc	Moderate
Arginine 0.2	1.42 ± 0.16 def	No	2.93 ± 0.21 a	Slight
Arginine 0.5	2.32 ± 0.16 a	No	1.41 ± 0.10 e	No
Arginine 1.0	1.59 ± 0.10 d	Slight	1.74 ± 0.15 cde	Slight
Arginine 2.0	1.31 ± 0.07 efg	Severe	1.70 ± 0.12 cde	Slight
Lysine 0.2	1.63 ± 0.07 cd	No	2.04 ± 0.11 bc	Slight
Lysine 0.5	2.21 ± 0.15 a	No	2.05 ± 0.12 bc	Slight
Lysine 1.0	1.97 ± 0.18 b	No	1.58 ± 0.10 de	Moderate
Lysine 2.0	1.48 ± 0.08 de	No	1.51 ± 0.15 de	Moderate

Table 5. Effects of amino acid combination treatments on *C. goeringii* rhizome proliferation.

Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation	Amino acids (mmol·L ⁻¹)	Proliferation rate	Browning status during proliferation
Cysteine 2.0 + proline 1.0	2.61 ± 0.19 c	No	Arginine 0.5 + proline 1.0	3.05 ± 0.43 a	No
Cysteine 2.0 + isoleucine 0.2	2.69 ± 0.24 c	No	Arginine 0.5 + isoleucine 0.2	2.29 ± 0.22 cd	No
Cysteine 2.0 + asparagine 0.2	2.53 ± 0.22 cd	No	Arginine 0.5 + asparagine 0.2	2.66 ± 0.36 bc	No
Cysteine 2.0 + threonine 0.2	2.32 ± 0.26 d	Severe	Arginine 0.5 + threonine 0.2	2.50 ± 0.28 bc	No
Cysteine 2.0 + glutamate 0.2	2.38 ± 0.26 d	Slight	Arginine 0.5 + glutamate 0.2	2.42 ± 0.27 c	No
Cysteine 2.0 + arginine 0.5	3.01 ± 0.27 a	No	Arginine 0.5 + cysteine 2.0	3.01 ± 0.27 a	No
Cysteine 2.0 + lysine 0.5	2.83 ± 0.29 ab	No	Arginine 0.5 + lysine 0.5	2.91 ± 0.26 ab	No

Table 6. Effects of amino acid combination treatments on *C. goeringii* rhizome differentiation.

Amino acids (mmol·L ⁻¹)	Differentiation rate	Browning status during differentiation	Amino acids (mmol·L ⁻¹)	Differentiation rate	Browning status during differentiation
Asparagine 0.5 + tyrosine 1.0	5.34 ± 0.25 a	Severe	Tyrosine 1.0 + aspartate 0.2	4.89 ± 0.18 b	Severe
Asparagine 0.5 + aspartate 0.2	5.39 ± 0.21 a	Moderate	Tyrosine 1.0 + cysteine 0.5	4.67 ± 0.15 c	Severe
Asparagine 0.5 + cysteine 0.5	4.95 ± 0.19 b	Moderate	Aspartate 0.2 + cysteine 0.5	4.78 ± 0.25 bc	Moderate

After treatment with amino acids, the differentiated buds can grow into seedlings suitable for transplanting out of bottles (Fig. 2F).

Discussion

Amino acids are important physiological active substances in plants that have an important role in regulating growth. We systematically investigated the effects of 20 amino acids on the proliferation and differentiation of *C. goeringii* rhizomes formed by aseptic seeding. Our results indicate that different types and concentrations of amino acids vary in their effects on the growth of *C. goeringii* rhizomes. The addition of cysteine, arginine, asparagine, proline, and lysine significantly promoted the proliferation of *C. goeringii* rhizomes without browning. Furthermore, arginine combined with cysteine or proline resulted in the highest proliferation rates of *C. goeringii* rhizomes. Conversely, the addition of amino acids during differentiation often caused browning. However, tyrosine, asparagine, and aspartate increased the differentiation rate of rhizomes, and asparagine combined with aspartate resulted in the highest differentiation rate.

Amino acids can promote the growth and development of plants, but excess concentrations inhibit growth (Chen et al., 2017). We found that the effects of many amino acids on the growth of rhizomes were in accordance with this rule. Increasing the content of cysteine in vivo can help improve the ability of plants to resist oxidative and environmental stress (Dominguez-Solis et al., 2004). This experiment showed that cysteine, alone or together with other amino acids, could promote rhizome growth. The reason for this may be that cysteine, as an antioxidant, can effectively inhibit the activities of catalase and polyphenol oxidase, thereby inhibiting browning and promoting rhizome growth. Lu et al. (2018) showed that arginine and lysine could promote rice growth and tillering bud elongation. During this experiment, arginine and lysine, alone or in combination, could promote rhizome proliferation. The reason may be that lysine can increase the activities of glutamic-oxaloacetic transaminase and alanine transaminase in plants and change the nitrogen metabolism of plants.

Plants mitigate stress by controlling the absorption, synthesis, and degradation of amino acids (Bottari and Festa, 1996). We found that asparagine can also alleviate rhizome browning during the proliferation stage and promote rhizome proliferation and differentiation. Proline can increase the adaptability of plants to external stresses, enhance or stabilize the activity of detoxifying enzymes, and stimulate the accumulation of

stress response proteins (Matysik et al., 2002). During this experiment, proline alleviated rhizome browning and promoted rhizome proliferation.

Many amino acids, including phenylalanine, resulted in *C. goeringii* rhizome browning and inhibited rhizome growth. It has been reported that phenylalanine can inhibit *Arabidopsis* seed germination and seedling growth (Voll et al., 2004), and high concentrations of phenylalanine inhibit rice growth (Lu et al., 2018). Therefore, our results are consistent with those of other studies. Tyrosine is an essential aromatic amino acid required for synthesis of proteins and diverse natural products (Schenck and Maeda, 2018). We found that tyrosine promotes rhizome differentiation, which has not been observed before.

In conclusion, arginine, lysine, proline, and cysteine, which mainly belong to polar basic amino acids (arginine and lysine) and neutral amino acids (proline and cysteine), are suitable for rhizome proliferation of *Cymbidium goeringii*. However, only polar neutral amino acids (asparagine or tyrosine) and polar acidic amino acid (aspartate) are suitable for rhizome differentiation of *Cymbidium goeringii*. In our study, one or two amino acids were investigated in each treatment. Therefore, it is unclear whether the addition of three or more amino acids together could further promote rhizome differentiation and proliferation. Future studies should investigate the effects of more amino acid combinations and other additives. Whether different amino acids can promote the seedling rooting of *C. goeringii* also needs to be investigated.

Literature Cited

- Bottari, E. and M.R. Festa. 1996. Asparagine as a ligand for cadmium (II), lead (II) and zinc (II). *Chem. Spec. Bioavail.* 8:75–83, doi: 10.1080/09542299.1996.11083272.
- Chen, Z.Y., T.P. Chen, H.J. Li, and S.Y. Chui. 2017. Research progress in molecular mechanism of amino acids absorption and transport by plants. *Mol. Plant Breed.* 15:5166–5171, doi: 10.13271/j.mpb.015.005166.
- Chung, M.Y. and J.D. Nason. 2007. Spatial demographic and genetic consequences of harvesting within populations of terrestrial orchid *Cymbidium goeringii*. *Biol. Conserv.* 137:125–137, doi: 10.1016/j.biocon.2007.01.021.
- Coruzzi, G. and D.R. Bush. 2001. Nitrogen and carbon nutrient and metabolite signaling in plant. *Plant Physiol.* 125:61–64, doi: 10.1104/pp.125.1.61.
- Dominguez-Solis, J.R., M.C. López-Martín, F.J. Ager, M.D. Ynsa, L.C. Romero, and C. Gotor. 2004. Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. *Plant Biotechnol. J.* 2:469–476, doi: 10.1111/j.1467-7652.2004.00092.x.
- Gao, R., S.Q. Wu, X.C. Piao, S.Y. Park, and M.L. Lian. 2014. Micropropagation of *Cymbidium sinense* using continuous and temporary airlift bioreactor systems. *Acta Physiol. Plant.* 36:117–124, doi: 10.1007/s11738-013-1392-9.
- Guha, S. and I.U. Rao. 2012. Nitric oxide promoted rhizome induction in *Cymbidium* shoot buds under magnesium deficiency. *Biol. Plant.* 56:227–236, doi: 10.1007/s10535-012-0081-7.
- Huang, W.T., Z.M. Fang, S.J. Zeng, J.X. Zhang, K.L. Wu, Z.L. Chen, A.T. Jaime, and J. Duan. 2012. Molecular cloning and functional analysis of three *FLOWERING LOCUS T (FT)* homologous genes from Chinese *Cymbidium*. *Intl. J. Mol. Sci.* 13:11385–11398, doi: 10.3390/ijms130911385.
- Huang, W.T., L.T. Zeng, B.W. Wu, and Z.M. Fang. 2017. Effects of different organic additives and sodium citrate combination on rhizomes proliferation of *Cymbidium goeringii*. *J. Anhui Agr. Univ.* 44:1112–1118, doi: 10.13610/j.cnki.1672-352x.20171214.010.
- Ichihashi, Y., Y. Date, A. Shino, T. Shimizu, A. Shibata, K. Kumaishi, F. Funahashi, K. Wakayama, K. Yamazaki, A. Umezawa, T. Sato, M. Kobayashi, M. Kamimura, M. Kusano, F.S. Che, M.O. Brien, K. Tanoi, M. Hayashi, R. Nakamura, K. Shirasu, J. Kikuchi, and N. Nihei. 2020. Multi-omics analysis on an agroecosystem reveals the significant role of organic nitrogen to increase agricultural crop yield. *Proc. Natl. Acad. Sci. USA* 117:17259, doi: 10.1073/pnas.1917259117.
- Kan, C.C., T.Y. Chung, H.Y. Wu, Y.A. Juo, and M.H. Hsieh. 2017. Exogenous glutamate rapidly induces the expression of genes involved in metabolism and defense responses in rice roots. *BMC Genomics* 18:186, doi: 10.1186/s12864-017-3588-7.
- Krapp, A. 2015. Plant nitrogen assimilation and its regulation: A complex puzzle with missing pieces. *Curr. Opin. Plant Biol.* 25:115–122, doi: 10.1016/j.pbi.2015.05.010.
- Lee, O.R., D.C. Yang, H.J. Chung, and B.H. Min. 2011. Efficient in vitro plant regeneration from hybrid rhizomes of *Cymbidium sinense* seeds. *Hort. Environ. Biotechnol.* 52:303–308, doi: 10.1007/s13580-011-0187-4.
- Liu, Y., H.L. Zhang, H.R. Guo, L. Xie, R.Z. Zeng, X.Q. Zhang, and Z.S. Zhang. 2017. Transcriptomic and hormonal analyses reveal that YUC-mediated auxin biogenesis is involved in shoot regeneration from rhizome in *Cymbidium*. *Front. Plant Sci.* 8:1866, doi: 10.3389/fpls.2017.01866.
- Lu, K., B.W. Wu, J. Wang, W. Zhu, H.P. Nie, J.J. Qian, W.T. Huang, and Z.M. Fang. 2018. Blocking amino acid transporter *OsaAP3* improves grain yield by promoting outgrowth buds and increasing tiller number in rice. *Plant Biotechnol. J.* 16:1710–1722, doi: 10.1111/pbi.12907.
- Matysik, J., A. Alia, B. Bhalu, and P. Mohanty. 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Curr. Sci.* 82:525–532, doi: 10.1038/416238a.

- Paek, K.Y. and T. Kozai. 1998. Micropropagation of temperate *Cymbidium* via rhizome culture. HortTechnology 8:283–288, doi: 10.21273/HORTTECH.8.3.283.
- Park, H.Y., K.W. Kang, D.H. Kim, and I. Sivanesan. 2018. In vitro propagation of *Cymbidium goeringii* Reichenbach fil. through direct adventitious shoot regeneration. Physiol. Mol. Biol. Plants 24:307–313, doi: 10.1007/s12298-017-0503-2.
- Ramya, M., P.M. Park, Y.C. Chuang, O.K. Kwon, H.R. An, P.M. Park, Y.S. Baek, B.C. Kang, W.C. Tsai, and H.H. Chen. 2019. RNA sequencing analysis of *Cymbidium goeringii* identifies floral scent biosynthesis related genes. BMC Plant Biol. 19:337, doi: 10.1186/s12870-019-1940-6.
- Ren, R., J. Gao, C.Q. Lu, Y.L. Wei, J.P. Jin, S.M. Wong, G.F. Zhu, and F.X. Yang. 2020a. Highly efficient protoplast isolation and transient expression system for functional characterization of flowering related genes in *Cymbidium* orchids. Intl. J. Mol. Med. 21:2264, doi: 10.3390/ijms21072264.
- Ren, R., Y.L. Wei, S. Ahmad, J.P. Jin, J. Gao, C.Q. Lu, G.F. Zhu, and F.X. Yang. 2020b. Identification and characterization of NPR1 and PR1 homologs in *Cymbidium* orchids in response to multiple hormones, salinity and viral stresses. Intl. J. Mol. Sci. 21:1977, doi: 10.3390/ijms21061977.
- Roy, J. and N. Banerjee. 2002. Rhizome and shoot development during *in vitro* propagation of *Geodorum densiflorum* (Lam.) Schltr. Sci. Hort. 94:181–192, doi: 10.1016/S0304-4238(01)00373-9.
- Schenck, C.A. and H.A. Maeda. 2018. Tyrosine biosynthesis, metabolism, and catabolism in plants. Phytochemistry 149:82–102, doi: 10.1016/j.phytochem.2018.02.003.
- Shimasaki, K. and S. Uemoto. 1990. Micropropagation of a terrestrial *Cymbidium* species using rhizomes developed from seeds and pseudobulbs. Plant Cell Tiss. Org. 22:237–244, doi: 10.1007/bf00033642.
- Tegeder, M. 2012. Transporters for amino acids in plant cells: Some functions and many unknowns. Curr. Opin. Plant Biol. 15:315–321, doi: 10.1016/j.pbi.2012.02.001.
- Voll, L.M., E.E. Allaire, G. Fiene, and A.P. Weber. 2004. The *Arabidopsis* phenylalanine insensitive growth mutant exhibits a deregulated amino acid metabolism. Plant Physiol. 136:3058–3069, doi: 10.1104/pp.104.047506.
- Wang, J., B.W. Wu, K. Lu, Q. Wei, J.J. Qian, Y.P. Chen, and Z.M. Fang. 2019. The amino acid permease 5 (OsAAP5) regulates tiller number and grain yield in rice. Plant Physiol. 180:1031–1045, doi: 10.1104/pp.19.00034.
- Xiang, L., Y. Chen, L.P. Chen, X.P. Fu, K.G. Zhao, J. Zhang, and C.B. Sun. 2018. B and E MADS-box genes determine the perianth formation in *Cymbidium goeringii* Rchb.f. Physiol. Plant. 162:353–369, doi: 10.1111/ppl.12647.
- Yang, F.X., G.F. Zhu, Z. Wang, H.L. Liu, Q.Q. Xu, D. Huang, and C.Y. Zhao. 2017. Integrated mRNA and microRNA transcriptome variations in the multi-tepal mutant provide insights into the floral patterning of the orchid *Cymbidium goeringii*. BMC Genomics 18:367, doi: 10.1186/s12864-017-3756-9.
- Yang, F.X., G.F. Zhu, Y.L. Wei, J. Gao, G. Liang, L.Y. Peng, C.Q. Lu, and J.P. Jin. 2019. Low-temperature-induced changes in the transcriptome reveal a major role of CgSVP genes in regulating flowering of *Cymbidium goeringii*. BMC Genomics 20:53, doi: 10.1186/s12864-019-5425-7.
- Zhang, J.X., X.L. Zhao, R.X. Tian, S.J. Zeng, K.L. Wu, A.T. Jaime, and J. Duan. 2019. Molecular cloning and functional analysis of three *CONSTANS-Like* genes from Chinese *Cymbidium*. J. Plant Growth Regul. 39(3):1061–1074, doi: 10.1007/s00344-019-10044-9.