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Appropriate Condition for Cluster Buds Induction of *Cremastra appendiculata*

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Abstract To screen for suitable conditions for cluster bud induction of *Cremastra appendiculata*. The aseptic germination protocorms of *C. appendiculata* seeds were used as explant materials to explore the effects of different basic media, types of plant growth regulators, anti-browning agents and their concentrations on the induction of cluster buds of *C. appendiculata*. The results showed that: 1/4MS medium was the suitable basic medium for cluster buds growing; when the concentration of plant growth regulator TDZ was 2.0 mg/L and the concentration of IAA was 0.2 mg/L, the induction rates of cluster bud were higher which are 72.58% and 55.63% respectively; when the anti-browning agent was PVP and its concentration is 2 000 mg/L, the induction rate of cluster buds was higher which is 79.90%. The suitable conditions for *C. appendiculata* cluster buds induction are: 1/4MS + TDZ 2.0 mg/L+ IAA 0.1 mg/L+ PVP 2 000 mg/L, and the induction rate can reach 81.16%. This provided a basis for establishing a rapid propagation technology system of *C. appendiculata* and realizing its large-scale artificial cultivation.

Keywords *Cremastra appendiculata*; Cluster bud; Induction conditio

Rhododendron (*Cremastra appendiculata*) is a perennial herb, belongs to *Cremastra genus* in the family of Orchidaceae. It has the functions of clearing away fever and detoxification, promoting blood circulation and relieving pain, eliminating swelling and resolving congestion or toxin accumulation in your body, etc. (Chinese Pharmacopoeia Commission, 2005, Chinese Pharmacopoeia, pp.19-20). Its market demand is large cause of its high medicinal value. However, due to the difficulty of sexual reproduction and the low asexual reproduction coefficient, the natural resources of *Cremastra appendiculata* are extremely scarce (Lv, 2004; Lv et al., 2018). In the study of artificial propagation, the rhizome nodes were longer and the induced emergence rate was lower by the way of protocorm multiplication and seedling differentiation, it is still difficult to solve the problem of rapid propagation (Mao et al., 2006; Zhang et al., 2006). Ye (2018) found that the anti-browning agents and their concentrations could induce the occurrence of cluster buds of *C. appendiculata*, and the reproduction coefficient of *C. appendiculata* was obviously increased. While the appropriate condition for cluster buds induction of *Cremastra appendiculata* was not further explored.

In order to screen for appropriate conditions for cluster bud induction of *Cremastra appendiculata*, the protocorms of *C. appendiculata* seeds in this study were used as explant materials to explore the effects of different basic media, types of plant growth regulators, and anti-browning agents. And through orthogonal experiments, the appropriate combination of induction conditions was obtained, which provided a basis for establishing a rapid propagation technology system of *C. appendiculata* and realizing its large-scale artificial cultivation.

1 Results and Analysis

1.1 Effects of different basic media for inducing cluster buds of *C. appendiculata*

With the decrease of macronutrients in the basic medium, the induction rate of cluster buds of *C. appendiculata* increase gradually (Figure 1A). At the 1/4 MS medium, the induction rate of cluster buds was 45.37% (Figure 1B). The induction rate of cluster buds in 1/2 MS medium was slightly less than 1/4, which was 37.83%. MS medium had the worst induction rate, which was 23.75% (Figure 1C).

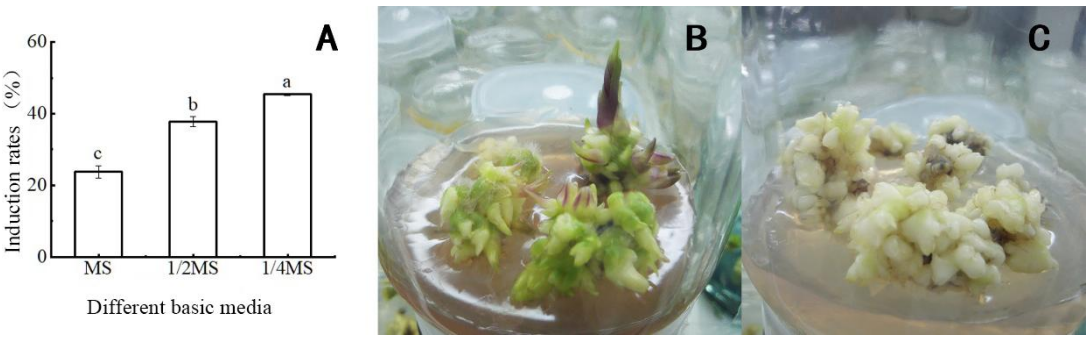

Figure 1 Effects of different basic media on the induction of cluster buds of *C. appendiculata*

Note: A: Effects of different basic media on the induction of cluster buds; B: 1/4 MS medium; C: MS medium; The different lowercase letter in the picture A indicate significant difference of $p<0.05$

1.2 Effects of cytokinins for inducing cluster buds of *C. appendiculata*

Different kinds of cytokinins have different promoting effects on the induction of cluster buds (Figure 2). Among them, TDZ induction effect was better, with the increase of TDZ concentration, the induction rate of *C. appendiculata* cluster buds showed the trend of first rising and then declining. When the TDZ concentration was 2.0 mg/L, the induction rate of cluster bud was the highest, which was 55.63%. TDZ concentration was 1.5 mg/L, the induction rate of cluster bud came second, which was 52.99%. When the TDZ concentration is 1.0 mg/L, 2.5mg/L, 3mg/L, the induction rate was about 35%, which was significantly lower than the first two concentrations.

The induction effect of 6- BA and KT on cluster buds of *C. appendiculata* was not significant. When the 6-BA concentration was 2.5 mg/L, the induction rate of cluster bud was the highest, which was 28.89%. When the KT concentration was 3.0 mg/L, the induction rate of cluster bud was 52.99%. But the induction rate with them was much lower than in the TDZ. With the increase of the concentration of 6-BA and KT, the induction rate of cluster bud also increased first and then decreased. Thus, TDZ is the more suitable cytokinin for inducing cluster buds of *C. appendiculata.*

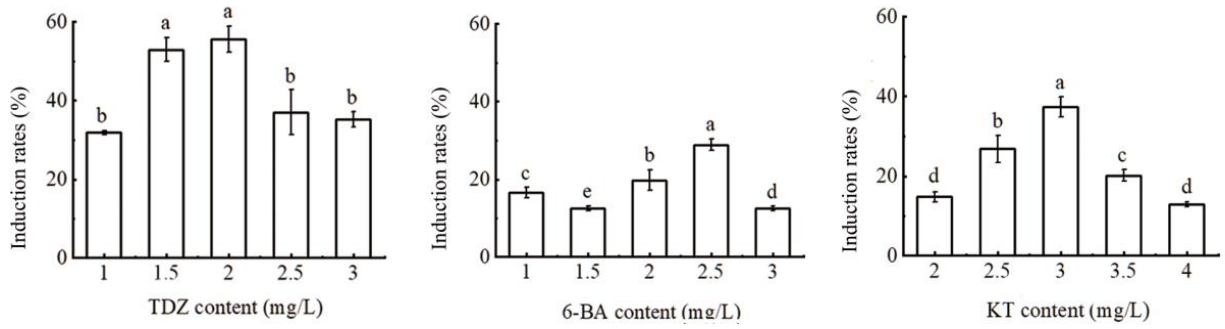

Figure 2 Effects of cytokinins for inducing cluster buds of *C. appendiculata*

Note: The different lowercase letter in the picture indicate significant difference of $p<0.05$

1.3 Effects of auxins for inducing cluster buds of *C. appendiculata*

During the experiment of different kinds and concentrations of auxin, the IAA effect was better (Figure 3A). When the IAA concentration was 0.2 mg/L, the cluster buds induction rate was 72.58%, which significantly higher than that with other concentration treatments. The induction rate under NAA treatment was lower than IAA, of which 0.2 mg/L NAA treatment had the highest induction rate of 37.44%, but the difference of cluster bud induction rate under different concentrations NAA treatment was not significant. In a word, IAA is more suitable for inducing cluster buds of *C. appendiculata.*

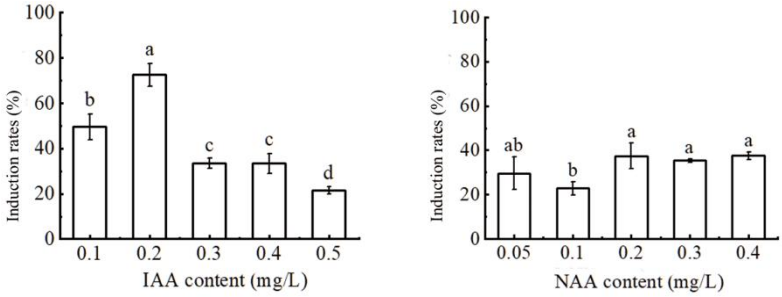

Figure 3 Effects of auxins for inducing cluster buds of *C. appendiculata*
Note: The different lowercase letter in the picture indicate significant difference of $p<0.05$

1.4 Effects of anti-browning agents for inducing cluster buds of *C. appendiculata*

Different kinds of anti-browning agents have different promoting effects on the induction of cluster buds (Figure 4). Among them, PVP induction effect was the best. When the PVP concentration was 2 000 mg/L, the induction rate was 79.9%. PVP concentration was 1 500 mg/L, the induction rate came second, which was 58.44%. The induction rate increased with PVP concentration. When the anti-browning agents were $Na_2S_2O_3$ and GSH, the induction rate of cluster buds increased first, and then decreased with the increase of concentration. The highest induction rate was 50.87% with 25 mg/L $Na_2S_2O_3$, while the induction rate was 34.27% with 25 mg/L GSH.

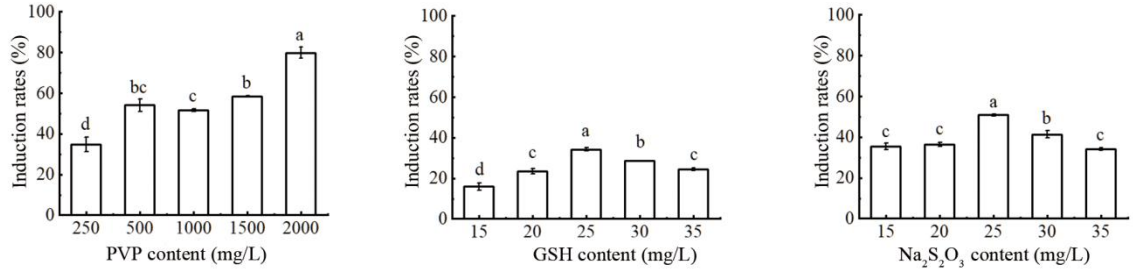

Figure 4 Effects of browning inhibitors for inducing cluster buds of *C. appendiculata*
Note: The different lowercase letter in the picture indicate significant difference of $p<0.05$

1.5 Effects of different factors interaction for inducing cluster buds of *C. appendiculata*

The interaction of macronutrients, plant growth regulators, and anti-browning agents had great influence on the induction of cluster buds of *C. appendiculata* (Table 1). In 9 treatments, the rate of cluster buds induction in No.8 was significantly higher than that in other treatments, which was 81.16%, followed by the No.6 and 9. But in No.6 and No.9 was much lower than in No.8 (Figure 5). The results of the range analysis showed that the maximum R of macronutrients was 38.08, which indicated that macronutrients had the greatest effect on the induction of cluster buds, followed by the TDZ (27.24), and the R of PVP was the minimum (4.34). Among the 4 factors, the order of influence on the cluster buds of *C. appendiculata* was macronutrients (38.08)>TDZ (27.24)>IAA (13.74)>PVP (4.34).

Macronutrients, plant growth regulators, and anti-browning agents had significant influence on the induction of cluster buds of *C. appendiculata* (Table 2). Cluster buds induction rate increased with the decrease of macronutrients, and when the base medium was 1/4 MS, the rate was higher. And the rate was obviously decreased when the base medium was MS, which indicated that the appropriate reduction of macronutrients was beneficial to the induction of cluster buds of *C. appendiculata.* At higher TDZ concentration, the cluster buds induction rate was higher. And with the decrease of TDZ concentration, the rate was also decrease. IAA had little effect on cluster buds induction, but when TDZ concentration is large, the lower the IAA concentration, the higher the cluster buds induction rate, which indicted the greater the ratio of TDZ to IAA concentration, the higher the cluster buds induction rate. PVP had the least effect on cluster buds induction, the induction rate was relatively high with a PVP of 2 000 mg/L. In a word, the appropriate conditions for *C. appendiculata* cluster buds induction are: 1/4 MS + TDZ 2.0 mg/L+ IAA 0.1 mg/L+ PVP 2 000 mg/L.

Table 1 Effects of different factors interaction for inducing cluster buds of *C. appendiculata*

No.	Main components	TDZ (mg/L)	IAA (mg/L)	PVP (mg/L)	Induction rates (%)
1	MS	1.5	0.1	1 000	10.69±0.45 i
2	MS	2.0	0.2	1 500	34.98±0.58 f
3	MS	2.5	0.3	2 000	20.04±0.36 h
4	1/2 MS	1.5	0.2	2 000	30.01±0.26 g
5	1/2 MS	2.0	0.3	1 000	45.94±0.77 d
6	1/2 MS	2.5	0.1	1 500	52.06±0.34 c
7	1/4 MS	1.5	0.3	1 500	39.69±0.45 e
8	1/4MS	2.0	0.1	2 000	81.16±1.46 a
9	1/4 MS	2.5	0.2	1 000	61.57±1.33 b
K_1	65.17	80.39	143.91	118.2	-
K_2	128.01	162.08	126.26	126.73	-
K_3	179.42	133.67	102.67	131.21	-
k_1	21.73	26.79	47.97	39.4	-
k_2	42.67	54.03	42.09	42.24	-
k_3	59.81	44.56	34.23	43.74	-
R	38.08	27.24	13.74	4.34	-

Note: The date of main components is the mean ± standard; lowercase letter significant difference of $p<0.05$; K is the sum of all factors at the same level, k is the average of all factors at each level

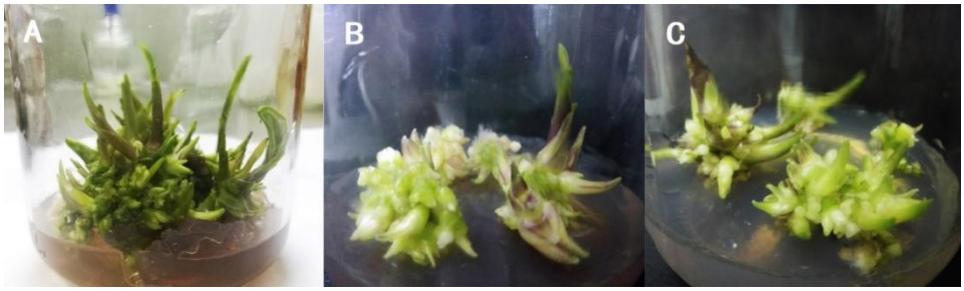

Figure 5 Effects of different treatment combinations for inducing cluster buds of *C. appendiculata*
Note: A: No. 8 processing; B: No. 6 processing; C: No. 9 processing

Table 2 Effects of different factors interaction for inducing cluster buds of *C. appendiculata*

Source	Type III square sum	Df	Mean square	F	Significance
Main components	6 836.610	2	3 418.305	109.557	0.000
TDZ	2 772.258	2	1 386.129	44.426	0.000
IAA	1 193.782	2	596.891	19.130	0.000
PVP	297.630	2	148.815	4.769	0.022
Error	561.618	18	31.201	-	-
Total	5 6355.389	27	-	-	-
Corrected total	1 1661.899	26	-	-	-

2 Discussion

The results showed that the 1/4MS medium was the best induction for cluster buds of *C. appendiculata.* High concentration of macronutrients in the MS medium could reduce the induction rate of cluster buds, and too high metal ion content could inhibit plant differentiation (Kundu et al., 2018). The existent results showed that reduce the concentration of macronutrients in the MS could significantly increase the induction rate of cluster buds. However, too few macronutrients will lead to undernutrition and growth arrest (Tan et al., 2013). And whether there is a lower or higher optimal concentration in the induction of cluster buds, the existing MS medium gradient needs to be refined. Besides, whether there are metal ion species in the MS which can inhibit the induction of cluster buds is worth further research.

Different kinds of cytokinins have different effects on the induction of cluster buds. TDZ has obvious inducing effect on cluster buds, compared with 6- BA and KT. Because TDZ belongs to phenylurea cytokinin in structure, it has better induction effect on buds in tissue culture of mature Orchidaceae than that of purine cytokinin (Sipayung et al., 2018). Joshi et al. (2008) found that the peanut somatic cell division ability was stronger when treated with high TDZ concentration, and the bud-like protuberance was more obvious, showing radial distribution.

In exploring the effects of IAA, TDZ and light factors on protocorm formation and seedling development of Orchidaceae plants, Vogel and Macedo (2011) found that the appropriate IAA concentration promoted the growth and differentiation of plant cell tissues and thus promoted the proliferation of protocorms, which is similar to the conclusion of this study that IAA can promote the growth of cluster buds. It found that IAA had better effect on cluster buds induction in this study, compared with NAA. This may be IAA metabolic rate faster than NAA, and the IAA indole ring occupies less space than the NAA naphthalene ring in the binding process (Singh et al., 2009). Thus, adding appropriate concentration of IAA could promote the induction of cluster buds of *C. appendiculata*.

Addition of appropriate concentration of anti-browning agents PVP could promote the induction of cluster buds. The main reason for plant browning is that phenols in plant tissues are oxidized by polyphenol oxidase to brown quinones, which inhibit other enzyme activities and affect plant growth (Saltiest et al., 2000). Metwally et al. (2003) found that some phenols play a regulatory role as signaling molecule in plant differentiation. PVP is an adsorbent of phenols and reduces the degree of plant browning by adsorbing specific species of phenolic compounds (Ye, 2018). Although phenols have an aggravating effect on plant browning, they are also necessary for plant growth. And PVP haven't fully adsorbed phenols in plants, cluster buds could grow better.

In order to screen for appropriate conditions for cluster buds induction of *Cremastra appendiculata*, we explore the effects of different basic media, types and concentrations of plant growth regulators, anti-browning agents on the induction of cluster buds of *C. appendiculata*. And obtained the appropriate conditions for *C. appendiculata* cluster buds induction are: 1/4MS + TDZ 2.0 mg/L+ IAA 0.1 mg/L+ PVP 2 000 mg/L. This improved the propagation coefficient for *C. appendiculata*, and provided an effective basic research and technical support for establishing a rapid propagation technology system. But this experiment didn't carry on the experiment such as rooting and seedling transplanting. It is necessary to further explore the chemical induction of cluster buds differentiation and growth of healthy plants to provide reliable basis for realizing its large-scale artificial cultivation.

3 Materials and Methods

3.1 Experimental materials

Cremastra appendiculata was collected from Shibing County, Qiandongnan, Guizhou Province (108°4"43" E, 27°0"26" N). After potted plants, seeds were obtained by artificial pollination, and protocorms were obtained by aseptic germination.

3.2 Effect of different factors on the induction of cluster buds

MS, 1/2MS and 1/4 MS were selected, added TDZ 2.0mg/L, NAA 0.2mg/L and sucrose 30 g/L to screen for appropriate conditions for cluster bud induction of *Cremastra appendiculata*. 1/4 MS + sucrose 30 g/L + NAA 0.2 mg/L as basic medium, experiment of single factor was set with different concentrations of cytokinin TDZ (1, 1.5, 2, 2.5, 3 mg/L), 6-BA (1, 1.5, 2, 2.5, 3 mg/L), KT (2, 2.5, 3, 3.5, 4 mg/L). 1/4 MS + sucrose 30 g/L + TDZ 2.0 mg/L as basic medium, experiment of single factor was set with different concentrations of auxin IAA (0.1, 0.2, 0.3, 0.4, 0.5 mg/L), NAA (0.05, 0.1, 0.2, 0.3, 0.4 mg/L). 1/4 MS + TDZ 2.0 mg/L + NAA 0.2 mg/L + source 30 g/L as basic medium, experiment of single factor was set with different concentrations of $Na_2S_2O_3$ (15, 20, 25, 30, 35 mg/L), GSH (15, 20, 25, 30, 35 mg/L) and PVP (250, 500, 1 000, 1 500, 2 000 mg/L). Above media were treated with pH 5.5~6.0, temperature (24±1)°C, light intensity 18.75~25μmol·m^{-2}·s^{-1}, and lighted 12 h/d. Each treatment was inoculated with 5 bottles, 7 protocorms per bottle. After 40 d, counted the cluster buds induction rate.

3.3 Screening for induction of cluster buds

Based on the above results of single factor experiments, orthogonal experiment was designed with basic medium (MS, 1/2MS, 1/4MS), plant growth regulator (TDZ, concentration of 1.5, 2, 2.5 mg/L; IAA, concentration of 0.1, 0.2, 0.3 mg/L) and anti-browning agents (PVP, concentration of 1 000, 1 500, 2 000 mg/L) according to $L_9(3^4)$ to screen the appropriate conditions for the induction of cluster buds of *C. appendiculata*.

3.4 Data statistics

Three or more buds per protocorm were as cluster buds

Cluster bud induction rate (%)=Number of protocorms producing cluster buds / Inoculated protocorms × 100.

3.5 Data analysis

All data were analyzed by SPSS 24 and mapped by Origin.

Authors' contributions

LSJ designed and carried out this experiment, and drafted the manuscript. GYY and YNX participated in the data analysis. TL and PSJ participated in the design of the study. ZMS guided the manuscript draft and revise. All authors read and approved the final manuscript.

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