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LATERAL BUD INDUCTION AND ESTABLISHMENT OF REGENERATION SYSTEM OF *CASSIA MIMOSOIDES* L.

YA-LI YANG[1], QIAN-QIAN DAI[1], PEI-TING HE[1], ZHAN-WEN LIN[1], SHENG-YUAN ZHANG[1], DA-DU LIN[1], YING LIU[2], MING-ZHAI[1]* AND LU-BIN ZHANG*

Guangdong Provincial Key Laboratory of Conservation and Precision Utilization of Characteristic Agricultural Resources in Mountainous Areas, Meizhou 514031, China

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Abstract

To establish an efficient plant regeneration system of *Cassia mimosoides*, the 10-day-old seedlings were used as explants for inducing lateral buds. Results showed that the addition of BA and NAA for different ratios on the basis of 1/2 MS could effectively induce the production of lateral buds. By adding different additional elements, it was found that the growth of lateral buds was longer and stronger on the medium of 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA + 4.0 mg/l Gln or 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA + 1.0 mg/l $AgNO_3$. Meanwhile, cutting with the lateral buds of tissue culture seedling, the rooting rate could reach 77.8% when 1/2 MS medium contained 0.2 mg/l NAA, although the root length was shorter. When the regenerated strong seedlings were transplanted into the soil, the survival rate was over 75%. Therefore, the best medium for lateral bud induction using 10-day-old seedlings as explants in *C. mimosoides* was 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA. The growth of lateral buds can be promoted by the appropriate concentration of Gln and $AgNO_3$, and the best rooting medium was 1/2 MS contained 0.2 mg/l NAA.

Introduction

Cassia mimosoides L., a leguminous plant belonging to Cassia, known as Mountain hyacinth bean, was first recorded in "Chiu Huang Pen Ts'ao" of the Ming Dynasty (Zhu *et al.* 2007). At that time, *C. mimosoides* was proved to be effective for heat-clearing and detoxifying, invigorating spleen to remove dampness and relaxing the bowels and so on (Song *et al.* 2001). Recent research further showed that *C. mimosoides* had good protection for the liver, due to its active ingredients such as flavonoids, quercetin, sterol and anthraquinone, etc (Han *et al.* 2016). So far, there are few studies on the pharmacodynamic activity of *C. mimosoides*. In the early days, scholars found that anthraquinone compounds in *C. mimosoides* could prevent and improve liver hypertrophy, body obesity and hypertriglyceridemia by its inhibitory effect on lipase (Subramanian *et al.* 1969). Then the water extract of *C. mimosoides* was proved to be effective in lowering fat and protecting the liver, which was consistent with the alcohol extract of *C. mimosoides* (Subramanian *et al.* 1970, Shen *et al.* 2015). In addition, similar studies also showed its protective effect on acute liver injury caused by alcohol or the hepatitis B virus (Zhang *et al.* 2009). Recent research further indicated that *C. mimosoides* may have the effects of scavenging free radicals by antagonizing lipid peroxidation, increasing the content of gSH-Px and GSH and decreasing the level of MDA so as to protect mitochondrial membrane and liver membrane (Lin *et al.* 2014, Liu *et al.* 2014).

Because of its good efficacy for the prevention and treatment of liver diseases, *C. mimosoides* is enjoyed and has a big market demand. However, the researches at home and abroad have been mainly focused on the chemical constituents and pharmacological studies of *C. mimosoides*. Therefore, it is necessary to develop and establish a tissue culture breeding system for rapid

*Author for correspondence: <yyali198622@163.com>,<rubzhang@126.com>; [1]School of Medicine, Jiaying University, Meizhou 514015, P.R. China. [2]College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, P.R. China

growth of *C. mimosoides*, in order to satisfy artificial cultivation and protect wild *C. mimosoides* resources. In earlier studies, a regeneration system was established by using young leaf explants to induce callus, which could shorten the culture cycles and the regenerated seedlings can grow well outdoors (Yang *et al.* 2020, 2021). The present study aims to establish a more efficient tissue culture regeneration system to solve the problem of long natural reproductive cycle time based on the previous study and will provide raw material support for further basic research on the activity analysis and liver-protecting application of *C. mimosoides.*

Materials and Methods

Seeds were adopted from *C. mimosoides* grown wildly in the Yinna mountain (24°39‘N, 116°39‘E) in Meizhou city of Guangdong Province and were surface sterilized and germinated on 1/2 MS medium (Murashige and Skoog 1962) supplemented with 0.8% agar and 1.5% sucrose. Seeds were treated at 80℃ for 5 min and surface sterilized before germinated on 1/2 MS with pH 5.8 (Liu *et al.* 2020, Yang *et al.* 2020). The 1/2 MS medium was sterilized in autoclave under conditions of 121℃ and 0.1 MPa for 20 min. The phytotron conditions were uniformly set at 22℃ under a 12-hour-light/12-hour-dark cycle, with light intensity at 2000~2500 lx and the relative humidity was 80%. NAA, BA, L-Glutamine (Gln), $AgNO_3$, Kinetin (KT), NaClO, sucrose, inositol and agar powder were used in the present study.

Ten-day-old seedlings of *C. mimosoides* were used as explants and were inoculated on 1/2 MS medium supplemented with different concentrations of BA and NAA. The length and the number of lateral bud induction were recorded after 50 days of culture. Based on the proper induction medium, different concentrations (0, 2, 4, 6 and 8 mg/l) of Gln, (0, 0.5, 1, 2 and 4 mg/l) of $AgNO_3$ and (0, 0.1, 0.5, 1 and 2 mg/l) of KT were added for lateral bud induction. Then the length of both pair of lateral buds was calculated at proper time.

Single buds with uniform growth and the length of about 2 cm were selected from the lateral bud induction medium after 60 days of induction. They were cut with scalpel and vertically inoculated into the rooting medium with different concentrations (0, 0.1, 0.2, 0.5 and 1 mg/l) of NAA on the basis of 1/2 MS basic medium. About 20 to 30 lateral buds were treated with each concentration. Data were collected 30 days later and the experiment was repeated for 3 times.

For data analysis, lateral bud induction rate = (number of seedlings producing lateral buds/number of connected stem segments) × 100%; Rooting rate = (number of adventitious roots/total number of inoculated lateral buds) × 100%; Rooting coefficient = (number of roots/number of rooting explants) ×100%; Transplanting survival rate = (number of surviving seedlings/total number of transplanted seedlings) × 100% were used (Yang *et al.* 2020). Data was processed and analyzed by Excel software and conducted with Duncan multiple comparisons ($P \leq 0.05$).

Results and Discussion

Results showed that the count of lateral buds after the treatment with NAA and BA was increased compared to the wide-type of *C. mimosoides* with only a pair of lateral buds. The combination of 3.0 mg/l BA and 0.1 mg/l NAA showed the best results that appeared 3 pair of lateral buds (Fig. 1A). Meanwhile, there was no obvious difference in the average length of the first two pair of lateral buds (Fig. 1B). These results indicated that both NAA and BA had good effect on lateral bud regeneration and the optimal concentration of BA was 3.0 mg/l, while NAA was 0.1 mg/l. Murashige and Skoog medium (MS) is the most widely used medium in the tissue culture of herbaceous plants, with sufficient contents of inorganic salts such as ammonium salts, nitrate salts and potassium salts and more trace elements. In the process of inducing the lateral

buds, the selection of the type and ratio of growth regulators is very important. In this study, the inducing rate of lateral buds was 100% by adding different ratio of BA and NAA hormones. 1/2 MS was chosen as the basic medium in terms of cost, combined with 3.0 mg/l 6-BA and 0.1 mg/l NAA. As plant hormones, both NAA and BA play a vital role in induction of callus information, lateral bud and adventitious bud regeneration, rooting and so on (Bao *et al.* 2004). Data further indicated that plant hormones perform various functions in the development of *C. mimosoides* propably because of the intersection between auxin and cytokinin signaling pathways.

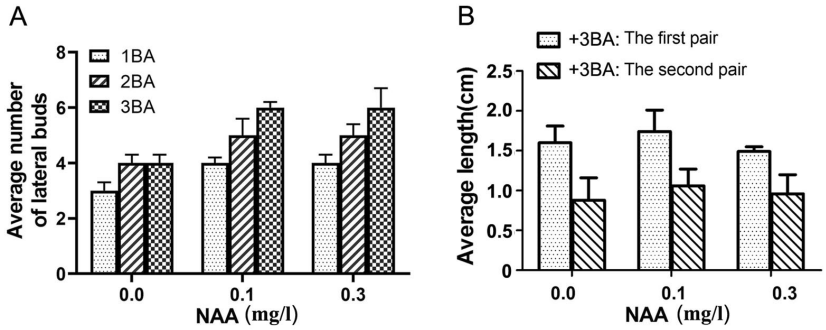

Fig. 1. Effects of NAA and BA on the induction of lateral bud in *C. mimosoides*.

Ten-day-old seedlings were inoculated on 1/2 MS medium contained BA and NAA for induction of lateral bud in *C. mimosoides* for 50 days. 1BA/2BA/3BA represents for 1.0/2.0/3.0 mg/l BA. A. The average number of lateral buds; B. The average length of the first two pair of lateral buds.

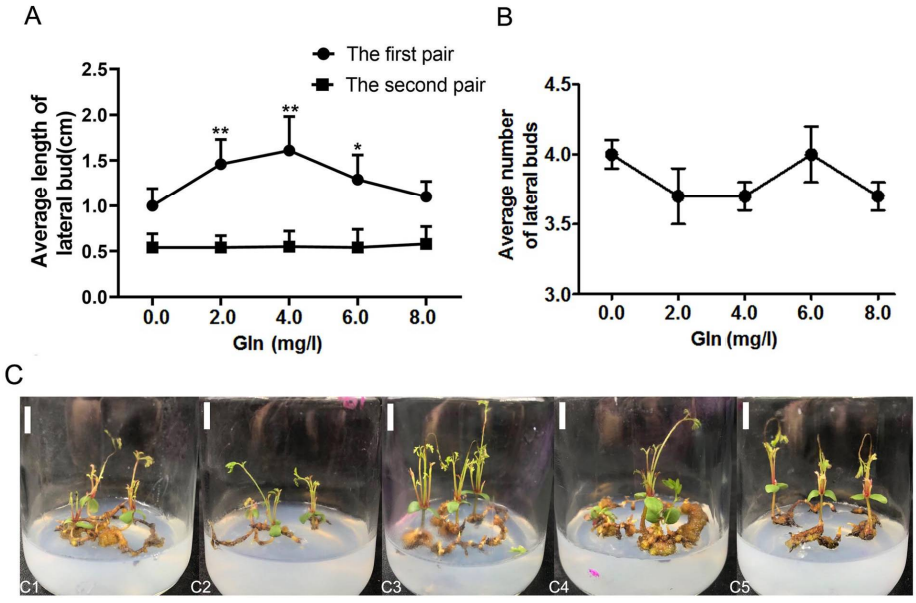

Fig. 2. Effects of Gln on growth of lateral buds development in *C. mimosoides*.

Ten-day-old seedlings were inoculated on 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA medium for induction of lateral bud supplemented with C1: 0 mg/l, C2: 2.0 mg/l, C3: 4.0 mg/l, C4: 6.0 mg/l and C5: 8.0 mg/l Gln

in *C. mimosoides* for 3 weeks. (Bars =1 cm) A. The average length of the first two pair of lateral buds B. The average number of lateral buds.Note: *represents for $P < 0.05$, ** represents for $P < 0.01$.

Glutamine (Gln) plays an important role in vital activities, associated with tissue growth and repair, including callus induction, bud proliferation and rooting as an organic nitrogen source (Harward *et al.* 1994). Different concentrations of Gln were added based on the medium of 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA for 3 weeks. Results showed that Gln could promote the growth of the first pair of lateral buds, while no obvious differences were found in the second pair of lateral buds (Fig. 2C) and 4.0 mg/l Gln was proved to be the most effective concentration for the first pair of lateral buds development (Fig. 2A), while there was no significant difference in the number of lateral buds (Fig. 2B). In conclusion, exogenous addition of Gln can promote the growth and development of the lateral buds, especially to the first pair. In the present study, Gln showed obvious acceleration on the growth of the first pair of the lateral bud, while no difference found in the second pair might be due to the short growth time.

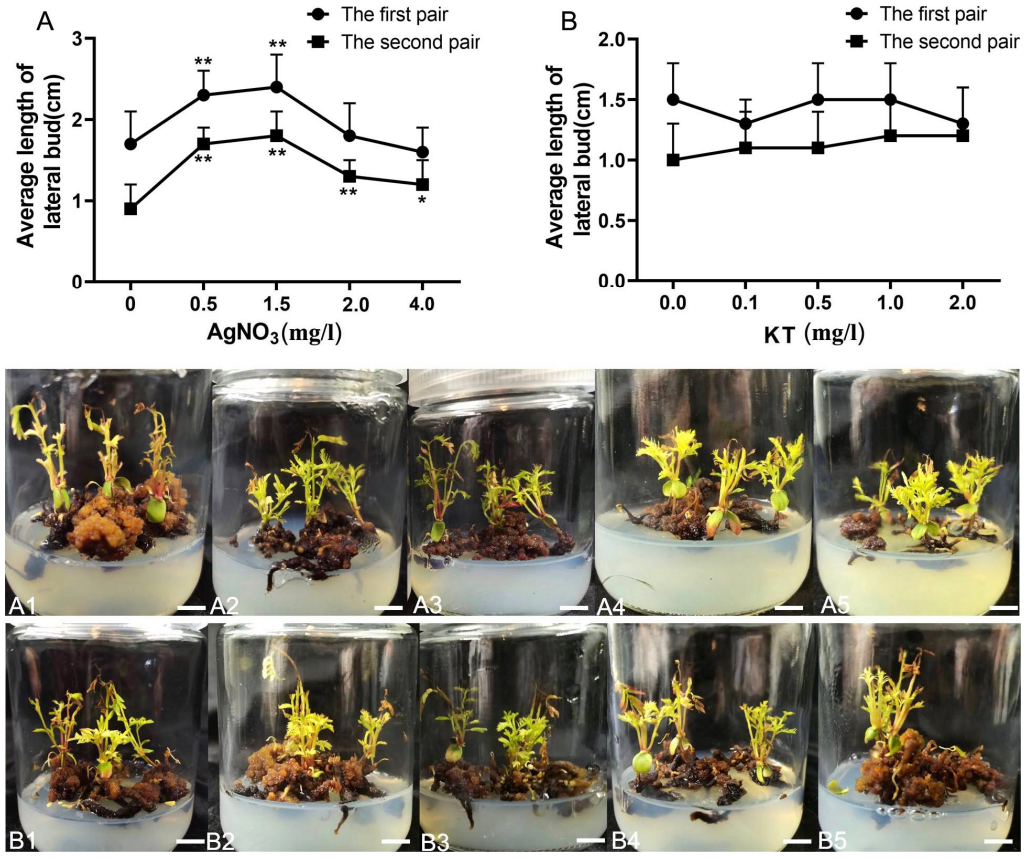

Fig. 3. Effects of $AgNO_3$ and KT on the development of lateral buds.

A: The average length of the first two pair of lateral buds supplemented with A1: 0 mg/l, A2: 0.5 mg/l, A3: 1.5 mg/l, A4: 2.0 mg/l and A5: 4.0 mg/l $AgNO_3$ in *C. mimosoides* for 50 days (Bars =1 cm). A. The average length of the first two pair of lateral buds supplemented with B1: 0 mg/l, B2: 0.1 mg/l, B3: 0.5 mg/l, B4: 1.0 mg/l and B5: 2.0 mg/l KT in *C. mimosoides* for 50 days. Note: * represents for $P < 0.05$, ** represents for $P < 0.01$.

Silver nitrate ($AgNO_3$) could promote ethylene production *in vitro* organogenesis by inhibiting ethylene action sites and was proved to be effective for bud induction and regeneration at proper concentration (Zhou and Zhuang 2002, Bednarek and Orłowska 2020). By adding various concentrations of $AgNO_3$, it was found that the lower concentrations of $AgNO_3$ (0.5 mg/l and 1.0 mg/l) could obviously promote the elongation of the both pair of lateral buds than the higher concentrations after 50 days cultivation (Fig. 3A). Data further showed the lower concentrations of $AgNO_3$ displayed a more pronounced stimulative effect on the second pair of lateral buds. These results revealed that the proper concentrations of $AgNO_3$ have good effect on the development of lateral buds. Here it was also found that $AgNO_3$ could effectively facilitate the growth of the lateral bud at 1.5 mg/l. The function of adding Ag^+ to culture medium is widely proved to be beneficial to the regeneration and the morphogenesis of adventitious buds of plants (Dowom *et al*. 2017). In *Solanum*, the addition of Ag^+ has good effect on the induction of adventitious shoots from axillary bud explants (Sridhar *et al*. 2011), result of which was in accordance with *Capsicum annuum* (Mythili *et al*. 2017). Recent study further showed that Ag^+ was proved to be useful for the induction of adventitious shoot regeneration in *C. mimosoides* (Liu *et al*. 2022).

Based on the basic lateral bud medium 1/2 MS + 3.0 mg/l BA + 0.1 mg/l NAA, different concentrations of KT were added. However, no significant changes were found in both pair of lateral buds after KT addition 50 days (Fig. 3B). To some extent, higher concentration of KT seems to be have an inhibition tendency for the first pair growth, which needs further study to prove. Recently study showed that lower concentrations of KT have a better proliferation effect on adventitious buds of *Populus hopeiensis* while higher concentrations of KT display the opposite effect (Wu *et al*. 2021). In the present experiment, the differentiation effect of KT on the lateral buds of *C. mimosoides* was not obvious, although it was demonstrated to be effective for the adventitious bud regeneration from the stem explants before (Liu *et al*. 2022). It might be because of the main effect of BA and NAA on the differentiation and regeneration of lateral buds, which superseded and covered the physiological effect of KT. Therefore, although exogenous elements have different degrees of promotion in the development of lateral bud, it is also necessary to select the proper composition rate.

For rooting, the lateral bud was then cut at about 2cm and inoculated vertically on 1/2 MS supplemented with NAA. The root was generated after 20 days and the longest root appeared in the medium without NAA (Table 1). However, the rooting rate changed with the increase of NAA concentration and up to a top at 0.2 mg/l. When the regenerated seedlings were transplanted into the soil, the survival rate was over 75%, which was counted after 20 days. Therefore, here one can choose 0.2 mg/l NAA as the proper rooting medium instead of 1/2 MS for the lateral bud. By adding different concentrations of NAA in the 1/2 MS, the rooting rate increased but the root length became shorter and shorter. In earlier studies, 1/2 MS was demonstrated to be better for the regeneration shoots from callus compared with NAA and IBA addition (Yang *et al*. 2020). Here, it was found that 1/2 MS medium contained 0.2 mg/l NAA which was more effective for rooting of lateral bud, suggesting that buds generated from different sites have different response to NAA.

In the present study, a more efficient regeneration system of *C. mimosoides* was established successfully. The 10-old-seedlings were used as explants instead of the callus or stem explants used before. Generally, buds induced from callus or seedling all could be used to root, according to the recent preliminary study on the regeneration system of *C. mimosoides* (Yang *et al*. 2021). Although a regeneration system used buds from callus have shortened the period of growing to at least 80 days (Yang *et al*. 2021), in the present study, a more effective way was explored by using buds from seedlings. To obtain a regenerated intact plant of *C. mimosoides*, the growth cycle has been shortened to more than 51 days by using this method. Furthermore, the induction rate of lateral bud was highly promoted by adding various elements than hormones only, which provides

a new effective way to obtain more regenerated plants from one seedling. Due to this efficient method, it will be easier to get materials for large scale planting. However, the effectively active components of regenerated plants of *C. mimosoides* changed or not are not clear. Therefore, it is essential to comprehensively explore the application prospect of tissue culture plants so as to make sure its quality for basic research as raw materials or mass industrial farming.

Table 1. Results of different concentration of NAA on rooting of lateral buds.

Concentrations of NAA (mg/l)	The average root length (cm)	Root coefficient	Rooting rate (%)
0	3.43 ± 0.26**	2 ± 0.35	33.3 ± 0
0.1	0.87 ± 0.11	1.9 ± 0.83	61.0 ± 13.4*
0.2	0.80 ± 0.06	3.2 ± 0.98*	77.8 ± 11.1*
0.5	0.50 ± 0.06	4.1 ± 1.58*	62.5 ± 9.9*
1	0.23 ± 0.05	4.6 ± 1.59*	58.3 ± 10.4

*represents for $P < 0.05$, **responds for $P < 0.01$.

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