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INFLUENCES OF VARIOUS FACTORS ON INDUCING ADVENTITIOUS SHOOT REGENERATION FROM STEM EXPLANTS OF CASSIA MIMOSOIDES LINN.

YING LIU*, YUAN-HANG ZHOU, ZI-XIN LIAO, HONG-JIE LIU, MIN ZHAO, JIAN-PING CHEN[1], YA-LI YANG[2] AND YING-BIN XUE*

Department of Biotechnology, College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China

Key words: Cassia mimosoides, Stem explant, Different factors, Plant regeneration, Rooting

Abstract

In the present study, an efficient plant regeneration method was established in *Cassia mimosoides* by using stem explants. Low regeneration frequency with poor quality advantageous shoot buds were obtained when the explants were grown only Kinetin (KN) or thidiazeuron (TDZ) enriched media. For improving the regeneration efficiency and production of good quality adventitious buds, Cu^{2+}, Mg^{2+}, Fe^{2+} and Ag^{+} were supplemented in the regeneration medium at different concentrations. The highest (63.96%) adventitious bud regeneration rate, the maximum number of buds (5.39) per explant and the longest regenerated buds (3.49 cm) were obtained by adding 26 mg/l Ag^{+} in the regeneration medium. In order to improve the rooting in shoot buds, diethyl aminoethyl hexanoate (DA-6) at different concentrations was added into rooting medium. The optimal rooting was obtained by supplementing 0.6 mg/l DA-6. The highest rooting rate (90.23%), the highest number of roots per bud (6.08) and the longest roots (4.26 cm) were developed within 20 days of culture in this medium. By applying this method culture cycle for obtaining complete regenerated plantlets was significantly shortened (in 60 days), Additional culture for proliferation and elongation of regenerated adventitious buds was not needed. Therefore, the method established in the present study was helpful to improve the efficiency of plant regeneration from stem explants in *C. mimosoides*. Besides the establishment of this culture method could lay a foundation for the future large-scale cultivation and genetic transformation of *C. mimosoides*.

Introduction

Cassia mimosoides is an important medicinal herb belonging to Fabaceae, native to China and grown as wild plant in many counties of the world. (Yang *et al.* 2020). It is located primarily in tropical and subtropical areas, and widely grown in southern China (Song *et al.* 2001, Fichadiya and Harisha 2017). Previous research results showed that many medicinal components such as emodin, oleanolic acid, β-sitosterol, luteolin, quercetin and so on are found in *C. mimosoides* (Shimura *et al.* 1993, Zhang *et al.* 2009, Han *et al.* 2016, Huang *et al.* 2017).

Results of research on the medicinal effects of the extract of *C. mimosoides* showed that extracts of *C. mimosoides* can significantly prevent and improve fatty liver, obesity and hypertriglyceridemia (Yamamoto *et al.* 2000). It was found that oleanolic acid can significantly promote *Nrf2* gene expression in liver cells of mice (*Mus musculus*) given alcohol, and inhibit aflatoxin-induced liver carcinogenesis by activating *Nrf2* (Wang *et al.* 2018). In other studies, the results revealed that the alcohol extract of *C. mimosoides* has a significant inhibitory effect on the formation of dimethylnitrosamine-induced liver fibrosis in mice, the mechanism of which might be due to its protective effect on liver cells (Han *et al.* 2016).

*Author for correspondence: <liuying85168@126.com>, <yingbinxue@yeah.net>. [1]Department of Food Science and Engineering, College of Food Science and Technology, Guangdong Ocean University, Zhanjiang 524088, Guangdong, P.R. China. [2]Provincial Key Laboratory of Conservation and Precision Utilization of Characteristic Agricultural Resources in Mountainous Areas, Jiaying University, Meizhou 514031, Guangdong, P.R. China.

By using the method of plant tissue culture, *C. mimosoides* plant materials can be obtained quickly and in large quantities. However, there are still some problems to be solved in the process of tissue-culture and fast asexual propagation of *C. mimosoides*. For example, the seed germination rate of *C. mimosoides* under natural conditions is extremely low, the efficiency of adventitious bud regeneration is not very efficient and remains to be improved and the cycle of plant regeneration is too long (at least 80 days) (Liu *et al.* 2020, Yang *et al.* 2021, Zhao *et al.* 2021).

In the present study a new tissue-culture regeneration system of *C. mimosoides* was established by evaluating the influence of various factors on the inducing effects of adventitious shoot regeneration from stem explants to shorten the culture cycle, improve the quality and regeneration efficiency for increasing the yield to meet the market demand, and provide technical support for the realization of the factory cultivation of *C. mimosoides*. Furthermore, it also laid the foundation for further research on the genetic transformation and gene function of *C. mimosoides*.

Materials and Methods

The seeds of *C. mimosoides* were collected from naturally grown plants in the Yinan mountain (24°39‘N, 116°39‘E) of Guangdong province of P. R. China (Yang *et al.* 2021). To get stem explants, the seeds were firstly immersed in water at 80°C for 10 min and then sterilized with the 2% sodium hypochlorite (NaClO) solution for 20 min, and finally washed with sterilized water for 5 times. The sterile seeds were placed onto MS medium (Murashige and Skoog 1962), kept in a culture room for 20 days to germinate. The stems were harvested from aseptic seedlings cut into small pieces (0.5 cm) and used as explant.

The cultivation media were supplemented with 2.5 % sucrose, 0.7 % agar and 100 mg/l inositol. The pH of the media was regulated to 5.8-6.0 with 1 mol/l NaOH solution. Afterwards, the media were sterilized in an autoclave for 20 min at 121℃ and 0.1 MPa. The culture conditions were maintained with stable illumination intensity (2000 Lux), constant temperature (25℃) and light duration (12 h/d) in the greenhouse.

To induce regeneration of adventitious shoots directly from the explants, stem explants were moved onto MS medium with the addition of different concentrations (0, 0.5, 1, 2 and 4 mg/l) of kinetin (KT) and various concentrations (0, 0.25, 0.5, 1 and 2 mg/l) of thidiazuron (TDZ) for 40 days of culture. To further explore the influence of different factors on adventitious bud regeneration, the stem explants were placed in regeneration medium (MS medium containing 2 mg/l KT and 0.5 mg/l TDZ) supplemented with various concentrations of $CuCl_2$ (0, 0.3, 0.6, 0.9 and 1.2 mg/l), $MgSO_4 \cdot 7H_2O$ (0, 0.25, 0.5, 0.75 and 1.5 mg/L), $FeSO_4 \cdot 7H_2O$ (0, 0.2, 0.4, 0.8 and 1.6 mg/l), and AgCl (0, 0.15, 0.3, 0.6 and 1.2 mg/L) for 40 days. The regenerated adventitious buds surpassing 1 cm in height were taken from mother materials, and then placed into rooting medium vertically (MS medium containing 0.1 mg/l indole 3-butyric acid (IBA)) with addition of various concentrations of 2-diethylaminoethyl hexanoate (DA-6) (0, 0.15, 0.3, 0.6 and 1.2 mg/l) for 20 days of culture.

In this study, all of the experimental processing was reproduced thrice, including 30 explants for each repetition. The test quantitative data were displayed as the mean ± standard deviation (SD) of the 3 repeated trials. All data were analyzed by SPSS 17.0 software for analysis of variance and Duncan’s multiple comparison ($P \leqslant 0.05$), and disparate letters on the top of histograms indicating significant differences among different treatments.

Results and Discussion

The regeneration of adventitious buds was induced by inoculating stem explants horizontally onto MS medium with various concentrations of KT (0, 0.5, 1, 2, and 4 mg/l). The experimental results indicated that as stem explants were moved on MS medium free of hormones, there was no bud regeneration (Fig. 1A-C and Fig. 2A). The most efficient concentration of KT was 2 mg/l, as reflected by the highest percentage of adventitious bud regeneration (25.93%), the maximum number of regenerative buds (2.37), and the longest of buds (0.76 cm) (Fig. 1A-C and Fig. 2B). However, as the KT concentration surpassed 2 mg/l, the adventitious bud regeneration rate was reduced significantly (Fig. 1A).

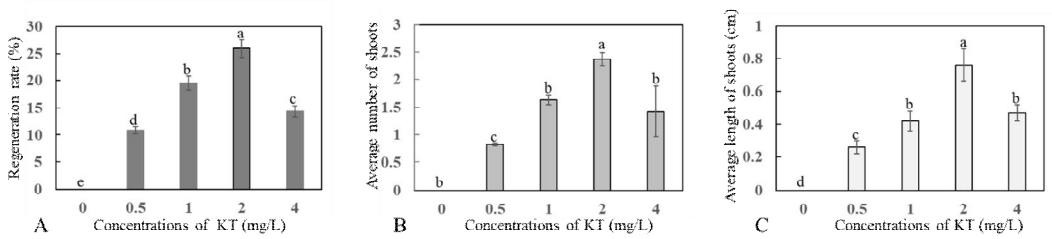

Fig. 1. Effect of KT at different concentrations on inducing regeneration of adventitious buds from stem explants in *C. mimosoides*. The stem explants were moved onto MS medium with various KT concentrations (0, 0.5, 1, 2 and 4 mg/l) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

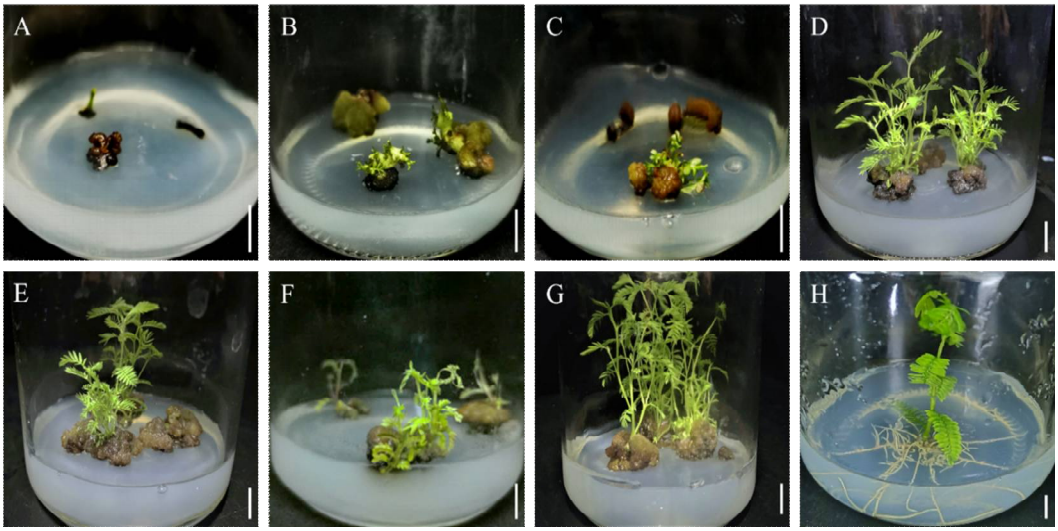

Fig. 2. Regeneration induction of adventitious shoots from stem explants of *C. mimosoides*. The stem explants were moved onto (A) MS medium; (B) MS medium with 2 mg/l KT; (C) MS medium contained 0.5 mg/l TDZ; regeneration medium with (D) 0.3 mg/l Cu^{2+}; (E) 0.5 mg/l Mg^{2+}; (F) 0.4 mg/l Fe^{2+}; (G) 0.3 mg/l Ag^{+} respectively for 40 days. (H) The regenerative buds were placed to rooting medium supplemented with 0.6 mg/l DA-6 for 20 days of culture. (Bars=1 cm)

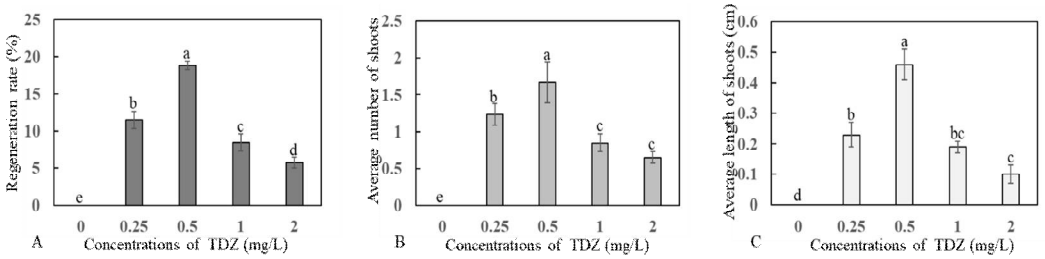

Fig. 3. Results of different TDZ concentrations on induction regeneration of adventitious buds from stem explants in *C. mimosoides*. The stem explants were placed onto MS medium with various TDZ concentrations (0, 0.25, 0.5, 1 and 2 mg/l) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

Stem explants were moved onto MS medium with the addition of different TDZ concentrations (0, 0.25, 0.5, 1 and 2 mg/l) for 40 days of culture. The results indicated that different TDZ concentrations obviously influenced the regeneration of adventitious shoots (Fig. 3A-C). When the TDZ concentration was 0.5 mg/l, the optimum regeneration rate (18.91%), the highest number (1.67) of regenerated buds and the longest adventitious buds (0.46 cm) were obtained (Fig. 2C and Fig. 3A-C). Nevertheless, when the working concentration of TDZ was more than 0.5 mg/l, an obvious inhibitory effect on shoot regeneration was observed (Fig. 3A-C).

In the present study, when only KT or TDZ was added to MS medium, poor regeneration results were obtained, and a lower adventitious bud regeneration rate and worse quality of regenerated buds were obtained (Fig. 1 A-C, Fig. 2 A-C and Fig. 3 A-C). Moreover, these regenerated buds were very small and difficult to continue to grow even after a long culture time (Fig. 2 B and C).

The traditional way to induce plant regeneration involves adding different hormones to the culture medium. KT and TDZ are two kinds of cytokinins commonly used in plant tissue culture (Hassan *et al.* 2018, Rohela *et al.* 2019, Wu *et al.* 2021). Results suggests that cytokinins can induce the formation of adventitious buds from plant explants (Feyissa *et al.* 2005, Savitikadi *et al*. 2020). The findings revealed that KT has a better proliferation effect on adventitious buds of *Populus hopeiensis* at lower concentrations, while, when the KT concentration too high, the proliferation rate of adventitious shoots will be reduced, and the growth status of regenerative shoots will become worse (Wu *et al*. 2021). In addition, it has been suggested that TDZ is a resultful plant growth regulator for inducing regeneration of bud, organogenic, and developmental pathways (Hassan *et al*. 2018). This could be accounted by the fact that endogenous plantgrowth-regulating compounds may be stimulated by TDZ in excised or intact tissues, and a similar auxin response or modification of endogenous auxin metabolism may also be imitated via TDZ (Sajid and Aftab 2009, Hassan *et al.* 2018). However, the results indicated that the effect of using KT or TDZ alone on inducing adventitious shoot regeneration of stem explants in *C. mimosoides* was not good in the present study. Furthermore, even when KT and TDZ were added simultaneously, the regeneration effect was not satisfactory. This might be because the stem explants were not sensitive to TDZ and KT and could not initiate the differentiation and dedifferentiation process quickly. The evidence shows that the regeneration effect of explants is different corresponding to various hormones, while the sensitivity of explants to different hormones is not all the same (Wei *et al.* 2004, Purkayastha *et al.* 2010).

To explore the effect of Cu^{2+} on regeneration induction of adventitious shoots, stem explants were moved onto regeneration medium (MS medium with the addition of 2 mg/l KT and 0.5 mg/l TDZ) supplemented with different concentrations of $CuCl_2$ (0, 0.3, 0.6, 0.9 and 1.2 mg/l). The results suggested that different Cu^{2+} concentrations significantly affected the regeneration effect of stem explants. As the Cu^{2+} concentration increased from 0 to 0.3 mg/L, the regeneration efficiency also increased from 28.22% to 55.56% (Fig. 4 A). When the concentration of $CuCl_2$ was 0.3 mg/l, the optimum regeneration percentage (55.56%), the maximum mean value of bud number (4.97) and the longest of regenerated buds (1.88 cm) were acquired (Fig. 2D and Fig. 4 A-C). In addition, when 2 mg/l KT and 0.5 mg/L TDZ were adopted together in the regeneration medium, the regeneration effect was better than that adding KT or TDZ lonely (Figs. 1, 3 and 4). However, with the addition of the right concentration of Cu^{2+}, the regeneration efficiency could be almost twice as high as that of the group without Cu^{2+}. Purnhauser and Gyulai 1993 the reported that shoot regeneration from wheat (*Triticum aestivum*) and triticale (*Triticosecale wittmack*) calli can be significantly enhanced by Cu^{2+}. Moreover, adding Cu^{2+} to the regeneration medium can improve the regenerative efficiency of *Jatropha curcas* explants (Varshney and Johnson 2010). Meanwhile, the findings of this study revealed that adding a suitable concentration of Cu^{2+} to the regeneration medium could indeed improve the regeneration effect of the stem explants of *C. mimosoides*. It might be that the electron transport chain can be affected by the addition of Cu^{2+}, and then influencing the synthesis and production of ethylene (Wang *et al.* 2002, Mansilla *et al.* 2018). Moreover, Cu^{2+} may be involved in forming complexes with ethylene (Bednarek and Orłowska 2020). There is evidence that the plant hormone ethylene participates in a large number of plant development processes, including the induction of adventitious buds and plant regeneration; for example, complete plantlet regeneration from the callus of barley (*Hordeum vulgare*) is significantly influenced by ethylene (Jha *et al.* 2007).

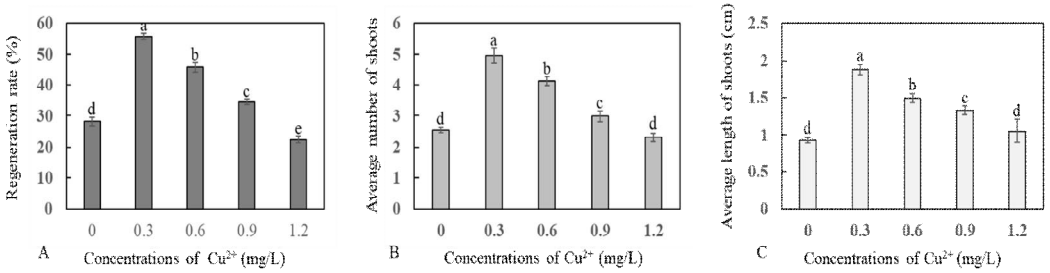

Fig. 4. Results of different concentrations of Cu^{2+} on induction regeneration of adventitious buds from stem explants of *C. mimosoides*. The stem explants were moved onto regeneration medium with various Cu^{2+} concentrations (0, 0.3, 0.6, 0.9 and 1.2 mg/L) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

To explore the effectiveness of Mg^{2+} on inducing regeneration of adventitious buds, stem explants were moved on regeneration medium containing different Mg^{2+} concentrations (0, 0.25, 0.5, 0.75 and 1.5 mg/l). As the concentrations of Mg^{2+} increased from 0 to 0.25 mg/l, the regeneration frequency also increased from 27.91 to 45.86% (Fig. 5A). It was found that the optimal regeneration concentration of Mg^{2+} was at 0.5 mg/l, and then the optimum regeneration percentage (52.68%), the maximum bud number (4.18) and the longest regeneration bud (1.51 cm) were subsequently obtained (Fig. 2E and Fig. 5 A-C). However, the induction frequency of adventitious buds was observably reduced, as the concentrations of Mg^{2+} exceeded 0.5 mg/l (Fig. 5 A-C). Wang *et al.* 2020 reported that using a suitable concentration of Mg^{2+} can promote the growth and production, t and improve the quality of *Blumea balsamifera*. The present findings

showed that when a suitable concentration of Mg^{2+} was added to the medium, the regeneration efficiency of *C. mimosoides* was improved to a certain extent. This might be due to a better material base for the growth and development of plants could be provided by adding Mg^{2+} into the medium. It was beneficial to the dedifferentiation of *C. mimosoides* explants into adventitious buds.

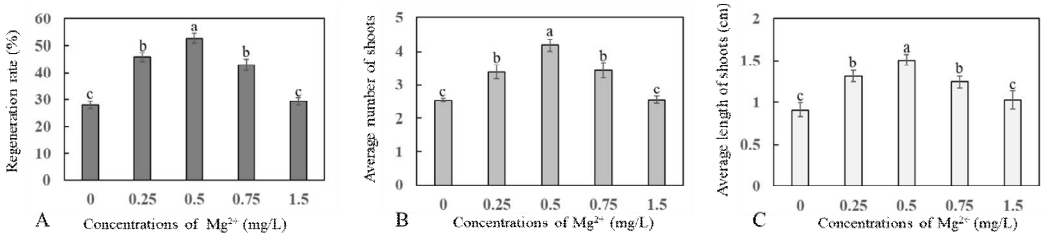

Fig. 5. Influence of different concentrations of Mg^{2+} on induction regeneration of adventitious buds from stem explants of *C. mimosoides*. The stem explants were placed onto re-generation medium with various Mg^{2+} concentrations (0, 0.25, 0.5, 0.75 and 1.5 mg/L) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

The influence of Fe^{2+} on the regeneration induction of adventitious buds from *C. mimosoides* stem explants was further analyzed. In the present study, stem explants were moved onto regeneration medium with the addition of different Fe^{2+} concentrations (0, 0.2, 0.4, 0.8 and 1.6 mg/l). As demonstrated in Figure 6, when the concentrations of Fe^{2+} increased from 0 to 0.2 mg/l, the regeneration rate increased from 28.17l to 36.17% (Fig. 6 A). The optimum Fe^{2+} concentration was 0.4 mg/l, at this concentration the highest regeneration rate (41.87%) (Fig. 6 A), the maximum bud number (2.96) (Fig. 6 B), and the longest shoots per explant (1.38 cm) (Fig. 6 C) were observed (Fig. 2F). However, the quality of regenerated adventitious buds was not as good and the leaves easily turned yellow and wilt (Fig. 2F). Furthermore, as the Fe^{2+} concentration exceeded 0.4 mg/L, the regeneration efficiency of adventitious shoots was notably hindered (Fig. 6 A-C). Few studies have focused on the role of Fe^{2+} in inducing the regeneration of adventitious buds from plant explants. The present study showed that although some positive effects on regeneration induction of adventitious shoots from stem explants of *C. mimosoides* were acquired under the condition of suitable Fe^{2+} concentration, the quality of regenerated buds was not as good. This might be due to excessive exogenous Fe^{2+} leading to excessive free Fe^{2+} in the plant, which would induce the generation of a variety of free radicals. While the accumulation of free radicals might lead to lipid peroxidation and membrane damage. Eventually, the growth of *C. mimosoides* adventitious shoots would be visibly affected.

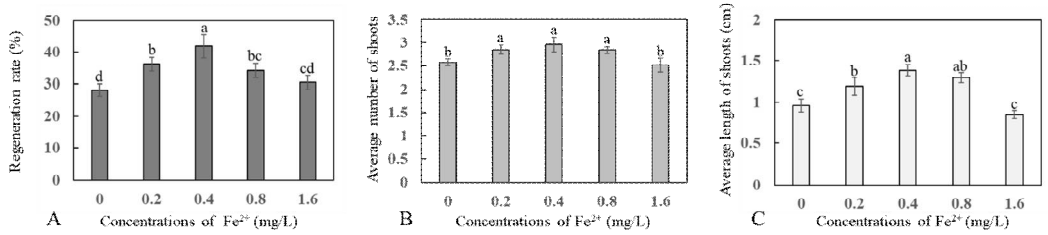

Fig. 6. Results of diverse Fe^{2+} concentrations on inducing regeneration of adventitious shoots from stem explants of *C. mimosoides*. The stem explants were placed to regeneration medium supplemented with various Fe^{2+} concentrations (0, 0.2, 0.4, 0.8 and 1.6 mg/l) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

To analyse the influence of Ag^+ on regeneration induction of adventitious shoots, the stem explants were moved onto regeneration medium horizontally for 40 days. The experimental results proved that diverse concentrations of Ag^+ prominently influenced the induction of adventitious shoot regeneration. As the Ag^+ concentrations increased from 0 to 0.15 mg/l, the rate of regeneration increased from 29.95 to 48.36% (Fig. 7 A). While the best result of inducing regeneration of adventitious shoots from stem explants was achieved by using 0.3 mg/l Ag^+, the regenerative percentage was 63.96%, the mean value of bud number was 5.39, and the mean value of bud length was 3.49 cm (Fig. 2 G and Fig. 7 A-C). Compared with several factors discussed previously, the optimum regeneration efficiency could be acquired when appropriate concentration of Ag^+ (0.3 mg/L) was used (Fig. 2 A-G and Fig. 1-7). Therefore, the most suitable culture condition was to add 0.3 mg/l Ag^+ to the regeneration medium.

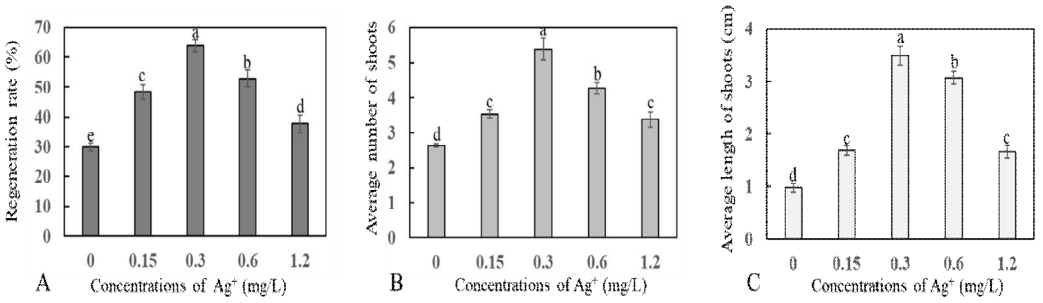

Fig. 7. Results of different concentrations of Ag^+ on induction regeneration of adventitious shoots from stem explants of *C. mimosoides*. The stem explants were moved onto regeneration medium with various Ag^+ concentrations (0, 0.15, 0.3, 0.6 and 1.2 mg/l) for 40 days of culture. A: Regeneration percentage of adventitious buds; B: Mean value of bud number; C: Average length of buds per explant.

Addition of different elicitors such as Ag^+ to culture medium is widely applied to accelerate the generation and amassing of secondary substances in the process of cultivation, which has a significant effect on the regeneration of plants (Wang *et al.* 2012, Dowom *et al.* 2017). By studying the effect of Ag^+ on the regeneration induction of adventitious shoots from axillary bud explants in *Solanum solanum*, it was found that Ag^+ was beneficial to the morphogenesis of adventitious buds (Sridhar *et al.* 2011). Furthermore, there is a synergistic response that further enhances the growth of regeneration buds of *Capsicum annuum* on the joint use of coconut juice and Ag^+ (Mythili *et al.* 2017). The height of shoots obtained from using Ag^+ synergistically with coconut water is higher than just adding Ag^+ alone (Mythili *et al.* 2017). The research findings demonstrated that the regenerated effect was improved significantly by adding an appropriate amount of Ag^+ to regeneration media for the induction of adventitious shoots from stem explants in *C. mimosoides*. The reason for this result might be that there was synergistic action for improving adventitious buds regeneration on the combined use of Ag^+ and cytokinins (KT and TDZ) in the regeneration medium, producing significantly better regeneration effect of adventitious bud when cytokinins were used synergistically with Ag^+ as opposed to cytokinins alone.

To explore whether DA-6 was beneficial to induce rooting of shoots, the regenerated shoots (greater than 1 cm in height) were taken from the parent explants and inserted vertically into the rooting medium supplemented with diverse DA-6 concentrations. As demonstrated in Fig. 8, the findings revealed that the induction rooting of shoots was significantly influenced by diverse DA-6 concentrations (Fig. 8 A-C). As the concentrations of DA-6 increased from 0 to 0.3 mg/l, the rate of rooting increased from 46.76 to 78.85% (Fig. 8 A). Furthermore, the production and the

development of roots were improved effectively with the addition of DA-6 to the rooting medium. The optimum concentration of DA-6 was 0.6 mg/l, and the best rooting percentage (90.23%), the largest number of roots (6.08) and the longest roots (4.26 cm) were acquired when the cultivation time was 20 days (Fig. 2 H and Fig. 8 A-C). Therefore, the best rooting condition was to add 0.3 mg/l DA-6 to rooting medium.

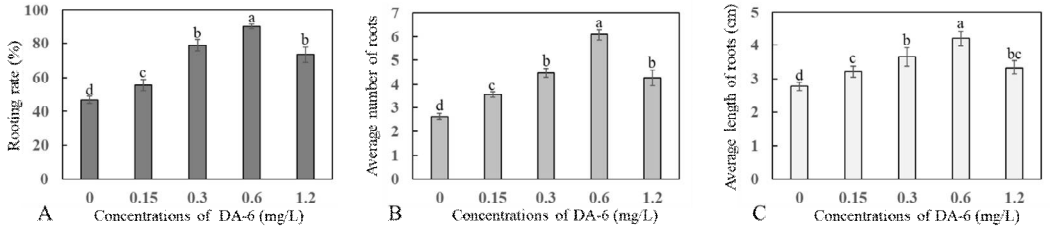

Fig. 8. Results of diverse DA-6 concentrations on inducing rooting from regenerative shoots of *C. mimosoides*. The regenerative shoots were placed into the rooting medium with various DA-6 concentrations (0, 0.15, 0.3, 0.6 and 1.2 mg/l) for 20 days of culture. A: Percentage of rooting; B: Mean value of root number; C: Mean value of root length.

Liu *et al.* (2021b) showed that when the DA-6 concentration was at 20 mg/l, the biomass of regenerated adventitious buds and roots increased by 27.55 and 37.46% respectively in grape (*Vitis vinifera*). In addition, studies have shown that DA-6 can accelerate the rooting of regenerated shoots in *Echinacea purpurea*, significantly improving root number and root length (Chen *et al.* 2013, Chen *et al.* 2016). The present findings indicated that the addition of a suitable concentration of DA-6 to the rooting medium was beneficial for improving the rooting effect of regenerated shoots of *C. mimosoides*. Once the DA-6 concentration exceeded a certain scope, the rooting of regenerated shoots was inhibited immediately. When DA-6 is applied at high concentrations, the growth of regenerated plants is hindered; however, the specific regulatory mechanism of DA-6 is not very clear (Chen *et al.* 2013, Chen *et al.* 2016).

In the present study, to set up a high-performance and short cycle regenerative method for *C. mimosoides*, many factors were discussed. The described new method of inducing adventitious buds from stem explants of *C. mimosoides* with added Ag^+ in regeneration medium might result in better regeneration results than the traditional method with using hormones only. Furthermore, because the raising output and improving quality of adventitious shoots were acquired by the innovative cultivation protocol in the present study, extra culture cycles of proliferation and elongation would not be needed, and therefore the time to obtain regenerated plants was reduced by at least 40 days. Yang *et al.* (2021) recently showed that it takes 80 to 120 days to obtain regenerated intact plants via the traditional method of adding only hormones into the culture medium. However, the period of regeneration culture would be reduced to less than 60 days by using the optimized method in this research. Because of the high regenerative efficiency of stem explants in *C. mimosoides*, it was easy to think of the application of *C. mimosoides* regeneration on *Agrobacterium*-mediated genetic transformation. This is because an efficient plant regeneration system is the premise of genetic transformation (Hayta *et al.* 2021, Thanh *et al.* 2021). Therefore, the high-efficiency plant regeneration method demonstrated in this study will likely be applied in breed improvement and research on gene function in *C. mimosoides.*

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