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Full Length Research Paper

Study of the system of tuberous root induction *in vitro* from *Rehmannia glutinosa*

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This study investigated the induction system of tuberous root *in vitro* from *Rehmannia glutinosa*. The roles of plant growth substance, carbohydrates, and minerals were evaluated for induction and development of tuberous root *in vitro*. The results show that Murashige and Skoog (MS) contributed greatly to induction of tuberous root *in vitro*, followed by α-naphthalene acetic acid (NAA), sucrose and 6-benzyladenine (BA). The optimal medium was ¼ MS supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA and 5% sucrose. In addition, paclobutrazol (PP_{333}) and methyl jasmonate (MeJA) also played an important role in induction of tuberous root *in vitro*, the most appropriate concentrations were 1 mgL^{-1} and 10 µmolL^{-1} respectively.

Key words: *Rehmannia glutinosa*, tuberous root, tissue culture *in vitro*.

INTRODUCTION

Rehmannia glutinosa, commonly known as "di-huang" in Chinese, is a perennial herb endemic to China, Japan and Korea. The tuberous root of *R. glutinosa* (the medicinal part of the herb) is very commonly used in Traditional Chinese Medicine in fresh, dried or steamed forms in terms of the processing methods. Traditionally, *R. glutinosa* has been used as tonics for replenishing Yin deficiency. Over the past two decades, considerable progresses have been made on the pharmacology of *R. glutinosa*. It has been found that *R. glutinosa* and its active components have wide pharmacological actions on the blood circulation, immune capability, endocrine balance, cardiovascular regulation and the health of nervous system (Zhang et al., 2008).

However, *R. glutinosa* was susceptible to virus as its roots were constantly used for propagation, thus the yield and quality of *R. glutinosa* are decreasing (Matsumoto et al., 1989; Wen et al., 2001). To solve the problem, there were attempts for *in vitro* approaches; Xu and Davey (1983) reported shoot regeneration from mesophyll protoplasts and leaf explants of *R. glutinosa*. Later, different culture conditions for *R. glutinosa* plantlets were investigated (Cui et al., 2000). Subsequently, Chen et al. (2004) reported callus induction and plant regeneration from various organs such as leaf, stem-segment, and petiole. Also, cultivation of virus-free seedlings could be a way for solving this problem (Shao et al., 2008), yet, it is difficult to apply it in agriculture production due to its unsatisfactory coefficient of propagation, long cycle of breeding and inconvenient transportation. In view of this situation, *in vitro* abnormal organs were successfully induced from potato (Sarkar et al., 2006) and yam (Olivier et al., 2011), which could substitute virus-free seedling in production.

Induction of tuberous root *in vitro* from *R. glutinosa* was also conducted (Xue et al., 2002), but the system of induction was imperfect. So far, the efficiency of induction on tuberous root *in vitro* could not achieve the desired yields.

As the average number of tuberous root obtained *in vitro* was small, the formation time was long, and the physiological mechanisms that controlled the morphogenesis of tuberous root were unclear. Therefore, it is necessary to search for an ideal system for high frequency tuberous root induction from *R. glutinosa*, which would also be a direct and convenient system for studying the mechanism that drives the formation of tuberous root.

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Table 1. The design form of the orthogonal test $L_9(3^4)$.

Level	Factor			
	A (MS)	B (BA, mgL^{-1})	C (NAA, mgL^{-1})	D (Sucrose, gL^{-1})
1	1/4	1.5	0.05	40
2	1/2	2.0	0.1	50
3	1	2.5	0.15	60

MATERIALS AND METHODS

Plant materials and culture conditions

Explants were collected from the tuberous roots of *R. glutinosa* grown at the experimental fields of Wenxian County, Henan Province, People's Republic of China (*R. glutinosa* was identified by Prof. Jianping Xue. A voucher specimen of the plant was deposited at the School of Life Sciences, Huaibei Normal University). They were cultured in sand at 25°C in light incubator. One week later, shoots formed in the tuberous roots were cut with one or two leaves, which were first washed thoroughly with tap water for 2 h. The shoots were immersed in 70% (v/v) ethyl alcohol and surface sterilized with 0.1% (w/v) mercuric chloride solution ($HgCl_2$) for 6 min, and then rinsed six times with sterile deionized water. The explants were sectioned from the sterilized shoots and incubated in 150 ml Erlenmeyer flasks at 2 to 3 explants per container each with 40 ml Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962), and supplemented with 1 mgL^{-1} BA and 0.1 mgL^{-1} NAA. The pH of the medium was adjusted to 5.8 before adding 7% (w/v) agar and autoclaved at 121°C for 15 min. Explants were incubated under 25 to 26°C and illuminated at 30 to 40 $\mu mol/m^{-2}s^{-1}$ with a photoperiod regime of 12 h light and 12 h dark cycle.

Tuberous root induction from *R. glutinosa*

One month later, seedlings cultured in the initial medium were cut into 2 to 3 cm long stems with one or two leaves and transferred to ½ MS medium supplemented with 1 mgL^{-1} indolebutyric acid (IBA) and 3% sucrose (Xue et al., 2002). Six days later, adventitious roots were formed in the seedlings, which were transferred to media with different treatments of orthogonal design when the length of adventitious roots was between 0.5 and 1 cm (Table 1).

Effects of paclobutrazol (PP_{333}) and methyl jasmonate (MeJA) on the induction of tuberous root

On the basis of the suitable medium for induction of tuberous root *in vitro* which was selected by orthogonal design, the effects of paclobutrazol (PP_{333}) and methyl jasmonate (MeJA) on the induction of tuberous root *in vitro* were studied respectively. With the same method, stems with adventitious roots were transferred to the media supplemented with different concentrations of PP_{333} (0, 1, 2, 3 mgL^{-1}) or MeJA (0, 0.1, 1, 10, 50 $\mu molL^{-1}$).

Statistical analysis

The experimental design was completely randomized with three replications per treatment, each treatment consisting of a total of 30 explants. Average weight of tuberous roots *in vitro* per seedling was recorded which were cultured for 20 days and the data were analyzed by using Minitab 15.

RESULTS

Effects of MS, sucrose, BA and NAA on induction of tuberous root *in vitro* from *R. glutinosa*

The *K* value (average of certain factor and level) can be used to determine the global optimal condition for induction of tuberous root *in vitro*. Experimental results showed that the *K* value by magnitude was $K_1>K_2>K_3$ (Table 2), which illustrated that ¼ MS played a good role in promoting root enlargement. With the increase of the concentration of major element in MS medium, the average weight of tuberous root per seedling decreased accordingly. On the other hand, as the concentration of NAA in this range (0.05 to 0.15 mgL^{-1}) increased, the average weight of tuberous root per seedling was enhanced; the average weight of tuberous roots *in vitro* formed in the medium supplemented with 0.15 mgL^{-1} NAA was 1.16 times as heavy as that in the medium supplemented with 0.05 mgL^{-1} NAA.

The *R* value (range of *K*) can show the effect of a certain factor on the tuberous root induction. Based on the *R* values, the effect orders of the four factors were not the same: *A*>*C*>*D*>*B*. The results of analysis of variance of average quality of tuberous roots per seedling among the four selected factors are presented in Table 3. Obviously, MS, NAA and sucrose all played significant roles in the formation of tuberous root *in vitro* due to *P*-values, which were all less than 0.05 but it was not significantly influenced by BA.

Comparing the *K* value of sucrose, K_2 was higher than the others, which showed that 5% sucrose was beneficial to the formation of tuberous root *in vitro*. Also, it indicated that sucrose promoted induction of tuberous root *in vitro* and development when its concentration increased from 4 to 5%, but an opposite effect appeared with the concentration of sucrose increasing between 5 and 6%.

With visual analysis according to Table 2, optimal medium for tuberous root induction from *R. glutinosa* could be obtained, which was $A_1B_1C_3D_2$: ¼ MS supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA and 5% sucrose

.This combination was not included in Table 2. 20 bottles of the medium from $A_1B_1C_3D_2$ were prepared for verification experiment. The average weight of tuberous roots per seedling was 0.1594 g and was slightly higher than those from nine combinations of the orthogonal

Table 2. Results of tuberous roots *in vitro* induced in different media of orthogonal design.

Number	Factors and their codes				Average fresh quality of tuberous roots per seedling (g)		
	A (MS)	B (BA, mgL^{-1})	C (NAA, mgL^{-1})	D (Sucrose, gL^{-1})	Replication 1	Replication 2	Replication 3
1	1/4	1.5	0.05	40	0.106	0.098	0.113
2	1/4	2.0	0.1	50	0.132	0.141	0.117
3	1/4	2.5	0.15	60	0.129	0.117	0.132
4	1/2	1.5	0.1	60	0.119	0.102	0.112
5	1/2	2.0	0.15	40	0.126	0.083	0.119
6	1/2	2.5	0.05	50	0.109	0.107	0.118
7	1	1.5	0.15	50	0.137	0.109	0.102
8	1	2.0	0.05	60	0.081	0.095	0.082
9	1	2.5	0.1	40	0.092	0.081	0.087
K_1	0.362	0.333	0.303	0.302			
K_2	0.332	0.325	0.328	0.357			
K_3	0.289	0.324	0.351	0.323			
R	0.073	0.009	0.048	0.055			

Table 3. Result of one-way analysis of variance.

Sources of variance	Sum of squares	df	F value	*P* value
A) MS	0.0025407	2	8.96	0.002**
B) BA	0.0000507	2	0.18	0.838
C) NAA	0.0013287	2	4.69	0.023*
D) Sucrose	0.0025513	2	4.90	0.020*
Error	0.0014105	18		

*Significantly different at $P<0.05$ among A, B, C and D; **significantly different at $P<0.01$ among A, B, C and D.

experiment, Figure 1a.

Effects of PP_{333} on the tuberous roots formation

Plantlets were transferred to the ¼ MS medium supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA, 5% sucrose and different concentrations of PP_{333} (0 to 3 mgL^{-1}) when the length of adventitious roots was between 0.5 and 1 cm.

Three days later, adventitious roots of the plantlet transferred to the medium without PP_{333} began to enlarge. Yet, the amount of time the adventitious roots became inflated in the medium supplemented with 1 to 3 mgL^{-1} PP_{333} was put off 7 days. With the concentration of PP_{333} increasing from 1 to 3 mgL^{-1}, the lagged effect on tuberous roots induction *in vitro* was visible. At 25 days, the tuberous roots induced in the medium without PP_{333} formed a small quantity of callus (Figure 1b) while that in the medium supplemented with PP_{333} continued expanding without callus. The medium supplemented with 1 mgL^{-1} PP_{333} was suitable for the formation of tuberous roots *in vitro* (Figure 1c); moreover, some golden tuberous roots with new buds were formed in this medium about two months later though plantlets almost died (Figure 1d, e).

Effects of MeJA on the tuberous roots formation

Plantlets were subcultured onto the ¼ MS medium supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA, 5% sucrose and different concentrations of MeJA (0 to 50 $\mu molL^{-1}$) when the length of adventitious roots was about 0.5 to 1 cm. Adventitious roots began to swell after 5 days in the media supplemented with MeJA with 0 to 10 $\mu molL^{-1}$. Significantly, more tuberous roots were observed in the medium containing 10 $\mu molL^{-1}$ MeJA after 10 days and no tuberous roots developed in the medium containing 50 $\mu mol.L^{-1}$ MeJA. When the concentration of MeJA increased to 10 $\mu molL^{-1}$, the average weight of tuberous roots was 5.5 times as heavy as that of the control group (Figure 1f, g). However, while the concentration of MeJA changed from 10 to 50 $\mu molL^{-1}$, a sharp decline trend could be observed according to Figure 2. Obviously, high concentrations of MeJA could

Figure 1. Development of tuberous roots of *R. glutinosa in vitro* in different media. A) tuberous roots of *R. glutinosa* formed in the screening medium: ¼ MS supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA and 5% sucrose; b) callus formed on the tuberous roots in the screening medium with further culture; c) tuberous roots formed in the screening medium supplemented with 1 mgL^{-1} PP_{333}; d and e) tuberous roots developed in the screening medium supplemented with 1 mgL^{-1} PP_{333} for two months; f and g) tuberous roots formed in the screening medium supplemented with 10 $\mu molL^{-1}$ MeJA.

inhibit tuberous root induction and development. Thus, among the five concentrations, medium supplemented with 10 $\mu molL^{-1}$ MeJA was the best one for tuberous root induction *in vitro*.

DISCUSSION

The metamorphosis of plant organs is a genetic trait, which largely depends on environment, plant growth substances, and nutritional conditions (Jose and Satheeshkumar, 2010). Usually, with cytokinin and auxin synergy, cells could divide rapidly and organ could be induced and developed. It was reported that no microtubers *in vitro* of *Dioscorea nipponica* could be induced in MS medium supplemented with either BA or NAA (Chen et al., 2007). In this study, the results show that NAA played a significant role in promoting tuberous roots formation *in vitro* and adventitious roots in the medium supplemented with BA but not NAA only elongated without swelling. It was reported that the pH value of cell wall matrix was reduced by auxin, so as to activate some enzyme (Hager et al., 1991), and the cell wall could be relaxed under the action of the enzyme, which would lead to cell expansion for organ development. As one of the auxins, NAA could also stimulate cell divisions. With increasing number and volume of cell, tuberous root generated naturally.

Major element concentration in MS medium plays an important role in the growth of plant *in vitro* and the synthesis and accumulation of metabolites, which could change ionic strength and osmotic pressure of the medium. In this trail, results show that adventitious roots in the ¼ MS medium swelled rapidly and the quality of tuberous roots formed in the medium was higher than those in the other media and there is a general agreement with the reports of Huang et al. (2010). However, it was the first time to study the effect of major element of MS on the induction of tuberous root *in vitro* and the concrete role of major element needs a further research.

In a similar manner to the phytohormones, sucrose has

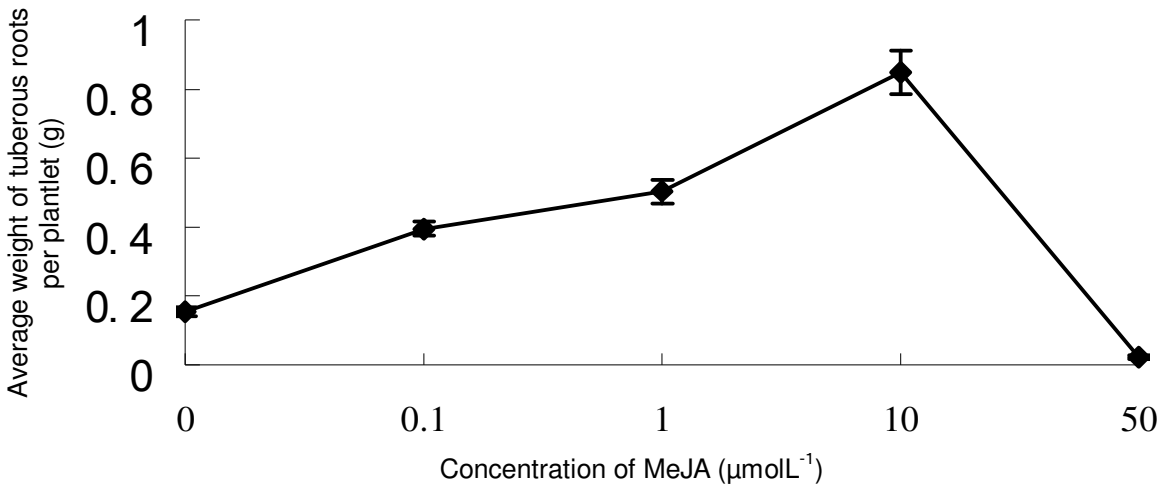

Figure 2. Effects of MeJA on the tuberous roots induction *in vitro*.

been shown to play an important role in the *in vitro* tuberous root formation of *R. glutinosa*. As a carbon source, sucrose could not only provide carbon for synthesis of new cell compounds, but also played a decisive role in regulating the water absorption of plantlets in tissue culture (Du et al., 2009). Indeed, higher sucrose concentration (8 to 12%) in growth media was shown to promote bulb induction (Zel et al., 1997) or potato tuberisation (Prat, 2010) and formation of tubers was delayed on media containing lower sucrose concentration in contrast to media with 3% sucrose as already observed by Lawrence and Barker (1963) on potato. Results of this experiment show that tuberous roots in medium containing 50 g/L sucrose developed well however, tuberous roots could not develop well in the medium either with low or high concentration of sucrose. Perhaps, low concentration of sucrose could not provide enough osmotic pressure for tuberous roots induction, and when the concentration of sucrose increased, it was difficult for plantlet to absorb water for the reduction of medium water potential, which was not conductive for photosynthesis. Therefore, the development of tuberous roots was negatively correlated with a lower or higher concentration of sucrose.

The previous studies suggested that gibberellic acid (GA_3) played a negative role in the formation of potato tubers *in vitro* (Hussey and Stacey, 1984). In this experiment, different concentrations of PP_{333} were added to the medium, which could inhibit the generation of endogenous GA_3, and the results showed that low concentrations of PP_{333} would be helpful in the formation of tuberous roots but high concentrations of PP_{333} would play a negative role in the development of tuberous roots. Our results were in partial agreement with indications given by Sheng et al. (1991), who noted that appropriate concentration of PP_{333} could promote tubers of potato formation and enlargement. Also, Zhang et al. (2004) pointed out that low concentration of PP_{333} promoted earlier potato formation, and increased microtuber fresh weight and mean diameter. It was reported that PP_{333} could increase chlorophyll content which could enhance leaf photosynthetic rate and inhibit the vegetative growth (Sheng et al., 1991). Meanwhile, PP_{333} inhibited plant producing endogenous GA_3. This could be the reason why appropriate concentration of PP_{333} would be facilitative for tuberous roots development. However, high concentration of PP_{333} would inhibit vegetative growth severely, which might inhibit tuberous roots development indirectly.

The trend was the same with PP_{333}. It could obviously be observed that low concentrations of MeJA promoted the development of tuberous roots while high concentrations of MeJA caused inhibition instead. It has been reported that appropriate concentrations of MeJA might increase the accumulation of storage material and then promote the development of tuberous root (Du et al., 2009). However, high concentrations of MeJA might promote premature aging of leaves (Chou and Kao, 1992; Chen et al., 2004), which would affect absorption of nutrients of plantlets and inhibit the enlargement of tuberous roots indirectly. Yet, the mechanism of MeJA on tuberous roots induction and development is still unclear and further studies in this direction would provide further insight into this aspect.

To sum up, the appropriate medium for tuberous root induction from *R. glutinosa* was ¼ MS supplemented with 1.5 mgL^{-1} BA, 0.15 mgL^{-1} NAA, 5% sucrose and 1 mgL^{-1} PP_{333} or 10 $\mu molL^{-1}$ MeJA. It was reported that GA and abscisic acid (ABA) was directly related to tubers development (Okazawa, 1960; Xu et al, 1998), and the influences of many other plant growth regulators on tubers mainly through an endogenous regulation of GA: ABA balance. We guess that the effects of those factors on tuberous root induction might depend on their roles in

regulating endogenous hormones, which might be related to regulation between vegetative and reproductive growth.

Conclusion

In conclusion, we studied induction of tuberous roots *in vitro* from *R. glutinosa* systematically and established an effective protocol for tuberous roots induction. It would provide materials for artificial seeds, which might be a new method for propagation of *R. glutinosa*. Meanwhile, it could be a new model for further studies on *R. glutinosa.*

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