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Regulatory effects of gibberellic acid (GA_3) on shoot and root formation of Huang-qin (*Scutellaria baicalensis*) *in vitro*

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Abstract: Influence of exogenously applied gibberellic acid(GA_3) on shoot and root formation and development in shoot tip and stem tissue cultures of Huang-qin(*Scutellaria baicalensis*), an important medicinal plant, was investigated. GA_3 application at the concentrations of 1-20 μmol/L resulted in the significant enhancement of shoot formation and growth while it reduced root formation and development. In different transferring tests, the exogenously applied growth regulators GA_3 and IAA resulted in significant changes in the concentrations of major flavonoids in Huang-qin tissues. The highest concentrations of baicalin, baicalein and wogonin were 14.90, 2.70 and 0.54 $\mu g \cdot mg^{-1}$ (dry weight), respectively, when the explants were pre-cultured on 2.5 μmol/L IAA medium for 6 days before transferred to MSO medium containing GA_3 at 5 μmol/L in continuous darkness.

Key words: adventitious rooting; Huang-qin; *Scutellaria baicalensis*; gibberellic acid; baicalin; baicalein and wogonin

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Huang-qin (*Scutellaria baicalensis* Georgi) is one of the most important medicinal plants used in traditional Chinese medicine (TCM). Its plants contain high levels of flavonoids, a group of low molecular weight polyphenolic compounds, which are considered to be the main medicinally active constituents of Huang-qin. Whole plant extracts and flavonoids of Huang-qin were reported to possess antiviral, anticancer, antibacterial, antimutagenic, antioxidative (Gao *et al.*, 1998), anti-inflammatory and anxiolytic activities (Hui *et al.*, 2002; Zhang *et al.*, 2003). Recent findings indicate that Huang-qin has melatonin and many other secondary metabolites with potential medicinal properties(Murch *et al.*, 1997; Pawlicki & Welander, 1992). Hence, Huang-qin is an ideal plant for scientific studies aiming to uncover novel active medicinal constituents.

Although it has been one of the major ingredients of TCM for over two thousand years, westerners' interest in Huang-qin is relatively recent. In East Asia, Huang-qin is generally wild harvested from nature. Wild harvest of medicinal plants presents many risks. Genetic differences among the plants, differences in the physiological stages at the time of harvest, contamination with biotic and abiotic agents, differences in harvesting and processing procedures and adulteration with other plant materials can result in inconsistency in active constituents, medicinal efficacy and even in serious toxicity(Li *et al.*, 2000). A recent study(Ye *et al.*, 2004) reported significant variation in chemical composition and biological activities of commercial Huang-qin extracts. Most of these problems can be solved by use of genetically uniform plant material in controlled production systems. *In vitro* propagation systems can be used as an alternative to wild harvest of this plant. A considerable amount of efforts has been made in developing *in vitro* propagation systems for Huang-qin by our group(Li *et al.*, 2000; Murch *et al.*, 1997, 2006; Zeng *et al.*, 2007; Zobayed *et al.*, 2004). However, an efficient, reproducible, high-frequency direct regeneration system has to be established to allow

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mainstream production of high quality plant material for the discovery of novel compounds, testing the medicinal efficacy and commercialization.

Gibberellic acid (GA_3) is a plant hormone of widespread occurrence in higher plants. It is involved in the control of number of plant growth and development responses. On one hand, gibberellic acid has been used in the microculture of plants for a number of purposes, including the stimulation of shoot proliferation in Malus(George & Sherrington, 1984) and *Atriplex canescens* (Wochok & Sluis, 1980). But it was found to inhibit shoot growth in *Ilex paraguariensis* (Sansberro *et al.*, 2001) and *Hancornia speciosa* (Pereira-Netto *et al.*, 2003). On the other hand, there is evidence for a positive effect of GA_3 on adventitious rooting in a number of species(Le *et al.*, 2001). In some cases its inhibitory effect has been reported (Pawlicki & Welander, 1992). Direct application of GA_3 tends to decrease rooting, and the application of inhibitors of GA biosynthesis can enhance rooting (Porlingis *et al.*, 1996). So there are different influences of GA_3 on shoot and root formations of different species. For these reasons, we investigated the effects of GA_3 on root morphogenesis and shoot formation of the germplasm line HQ_{111} from Huang-qin. The role of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and thidiazuron (TDZ) during different stages of root and shoot development has been reported as critical in Huang-qin(Li *et al.*, 2000; Zeng *et al.*, 2007). However, we do not have a complete understanding of whether other phytohormones including GA_3 play a role during root and shoot induction and/or development as well.

The present research is aimed at finding a reproducible method for *in vitro* multiplication of Huang-qin through shoot proliferation and rhizogenesis from shoot tip and stem explants of HQ_{111}. The tissue cultures were also examined for the ability to produce major flavonoids compounds.

1 Materials and methods

1.1 Plant material

Huang-qin line, HQ_{111}, was selected because of its high regeneration capacity *in vitro* and well studied chemical profile (Murch *et al.*, 2004). The plants were maintained aseptically for about 5 years through subculturing of shoot explant in MSO containing MS salts, B5 vitamins, 30 g · L^{-1} sucrose, and 3 g · L^{-1} gellan gum (Gelrite, Schweitzerhall, South Plainfield, NJ, USA) in about every two months. All cultures were incubated in a growth room with a 16 h photoperiod under cool-white light (30～40 μmol/L · m^{-2} · s^{-1}) at 25 ℃. Stem and shoot tip were used as explants. Stem explants (about 1.5cm) were prepared by cutting a node and excising the leaves, and shoot tip explants (1.5-2 cm) were prepared by cutting a node and the tip with intact leaves.

1.2 Treatment

A two-step culture for shoot or root formation was followed in the experiments. Seven explants were cultured on a Petri dish containing 25 mL of MSO medium supplemented with 2.5 μmol/L IAA or 2.5 μmol/L TDZ for 6 days. They were then transferred to a plate with MSO medium.

GA_3 (1-20 μmol/L) was added to 2.5 μmol/L IAA or 2.5 μmol/L TDZ or MSO medium and the explants were cultured on the both media without GA_3 as the controls. After 30 day incubation, the number of shoots or roots per explant was determined. The explants were cultured on MSO medium for all 30 days (hereafter referred to as MSO control) and those on MSO medium with 2.5 μmol/L TDZ or 2.5 μmol/L IAA (hereafter referred to as TDZ-treated controls or IAA-treated controls) for all 30 days.

1.3 Flavonoid analysis

Plantlets developed from both stem and shoot tip explants were collected after 30 days of culture and dried at 37 ℃ for 2 days in a drying chamber, then ground into fine powder and transferred into amber-colored 20 mL vials. The weight of the plantlets in the identical treatment was recorded before or after drying. Analytical methods for quantification of baicalein, baicalin and wogonin were made according to previously described methods by Murch (Murch *et al.*, 2004).

1.4 Statistical analysis

In each experiment, the treatments consisted of

three replications, and the experiments were repeated at least two times. Data were analyzed using the Statistical Analysis System (SAS Institute Inc., 2004) with Student-Newman-Keuls means separation test ($P \leqslant 0.05$).

2 Results and discussion

2.1 Effect of GA_3 on shoot induction and differentiation

Shoot induction of HQ_{111} was achieved by pre-incubating explants on 2.5 μmol/L TDZ medium for 6 days and subsequently culturing them on MSO medium for growth and development of shoots for 24 days. The addition of GA_3 to both media significantly stimulated shoot formation. GA_3 at concentration as low as 1 μmol/L caused about 1.3- and 0.2-fold increases of the control in shoot number per tip and stem explant, respectively, when added to 2.5 μmol/L TDZ medium. Approximately 3.0- and 5.0-fold increases of the control were obtained, respectively, when added to the MSO medium. GA_3 added to 2.5 μmol/L TDZ medium was similarly effective on shoot number per tip explant over all the concentrations tested, but the number of shoots per stem explant increased with increasing concentrations of GA_3 until it reached a maximum at 5.0 μmol/L and then decreased. When GA_3 added to MSO medium, the shoot number per explant reached a maximum at 1.0 μmol/L and then decreased. In addition, there was no clear difference between shoot number per explant cultured on the control, 2.5 μmol/L TDZ and MSO control media (Table 1).

Our group has demonstrated previously (Li *et al.*, 2000) that TDZ effectively induced shoot regeneration on cultured intact seedlings, etiolated hypocotyl explants and sterile stem segments of Huang-qin and adventitious shoots formed through an intermediate callus. The present experiment found that a similar adventitious shoots were induced by TDZ, but TDZ did not stimulate shoot formation on cultured sterile tip and stem segments of HQ_{111} (data no shown). The maximum shoot induction was observed at 2.5 μmol/L TDZ concentration for the explants of HQ_{111} (data no shown). This is presumably due to differences between the different Huang-qin germplasm lines examined. And exogenesis GA_3 promoted shoot formation and inhibited the callus formation of HQ_{111}. The stimulatory effect of GA_3 was more pronounced when used during the period of MSO medium than during the initial 6 days of 2.5 μmol/L TDZ medium (Table 1). Inhibition of callus formation by GA_3 has been reported previously by Ruduš (2002). The observation of inhibition of shoot multiplication and growth from the axillary meristem of the explants during an initial 6 days (Table 1) could be due to inhibition of TDZ on the development of axillary primordium. After the initial 6 days, shoots had developed to a point where TDZ was unable to inhibit the shoot formation from axillary primordium.

2.2 Effect of GA_3 on root induction and differentiation

The explants were pre-incubated on 2.5 μmol/L IAA medium for 6 days and subsequently cultured on MSO medium for 24 days. The addition of GA_3 to both media significantly inhibited root formation (Table 2). GA_3 at the concentration as low as 1 μmol/L reduced root number per tip and stem explant to about 60% and 53%, respectively, in comparison with the control when added to 2.5 μmol/L IAA medium and about 48% when added to MSO medium. The number of roots per explant decreased with increasing concentrations of GA_3 but no clear trend was evident when added to 2.5 μmol/L IAA medium. GA_3 added to MSO medium had a similar influence on root formation in all concentrations tested (Table 2).

There is considerable evidence that IAA with maximum enhancement was observed at 2.5 μmol/L concentration can promote root regeneration of explants of HQ_{111} (Zeng *et al.*, 2007). In contrast to its positive effect on shoot formation, exogenous gibberellin A3 significantly inhibited root growth (Table 2). Root growth was inhibited to a lesser extend if applied during MSO medium in stead of during 2.5 μmol/L IAA phase. GA_3 had an inhibitory effect after 6 days of the culture, so the roots from the explants could be induced by IAA before 6 days. This may suggest that the level of endogenous GA_3 is sufficient for root growth.

Table 1 Influence of GA_3 applied on shoot growth of HQ_{111} tissue culture in light

Treatment	Number of shoots per explant		Content of flavonoids		
	Tip	Stem	Baicalein ($\mu g\ mg^{-1}$, dry wt)	Baicalin ($\mu g\ mg^{-1}$, dry wt)	Wogonin ($\mu g\ mg^{-1}$, dry wt)
1 μmol/L GA_3	3.0476 a	2.4762 bcde	—	—	—
MSO	0.8571 b	1.4286 cdef	0.187 b	3.34 i	0.039
2.5 μmol/L TDZ	1.1905 b	1.2857 def	0.198 a	6.01 e	0.035
2.5 μmol/L TDZ[a]/MSO	0.9048 b	0.7619 ef	0.084 c	11.8 a	Trace
2.5 μmol/L TDZ[b]/1 μmol/L GA_3	3.6190 a	4.5714 a	0.045 g	5.98 e	Trace
2.5 μmol/L TDZ[b]/5 μmol/L GA_3	3.5238 a	4.000 ab	0.039	5.29 g	Trace
2.5 μmol/L TDZ[b]/10 μmol/L GA_3	2.8095 ab	2.7619 bcd	0.043 g	5.46 f	Trace
2.5 μmol/L TDZ[b]/20 μmol/L GA_3	2.2381ab	3.0952bc	0.028 h	3.74 h	ND
2.5 μmol/L TDZ +1 μmol/L GA_3[c]/MSO	2.0476 ab	0.9048 ef	0.088 c	7.54 c	Trace
2.5 μmol/L TDZ +5 μmol/L GA_3[c]/MSO	2.4286 ab	2.4286 bcde	0.068 e	7.41 d	Trace
2.5 μmol/L TDZ +10 μmol/L GA_3[c]/MSO	2.5238 ab	1.4286 cdef	0.052 f	9.51 b	Trace
2.5 μmol/L TDZ +20 μmol/L GA_3[c]/MSO	2.5238 ab	0.5238 f	0.074 d	7.46 d	Trace

1 μmol/L GA_3: Explants were cultured on 1 μmol/L GA_3 medium for 30 days; 2.5 μmol/L TDZ: Explants were cultured on 2.5 μmol/L TDZ medium for 30 days; MSO: Explants were cultured on MSO medium for 30 days. [a] Explants were pre-cultured on 2.5 μmol/L TDZ medium for 6 days before transferred to MSO medium. [b] Explants were pre-cultured on 2.5 μmol/L TDZ medium for 6 days before transferred to MSO medium containing GA_3 at the concentrations of 1,5,10 and 20 μmol/L. [c] Explants were pre-cultured on 2.5 μmol/L TDZ medium containing GA_3 at the concentrations of 1,5, 10 and 20 μmol/L for 6 days before transferred to MSO medium. Data recorded after 30 days of culture.

Table 2 Influence of GA_3 applied on root growth of HQ_{111} tissue culture in continuous darkness Treatment

Treatment	Number of shoots per explant		Content of flavonoids		
	Tip	Stem	Baicalein ($\mu g \cdot mg^{-1}$, dry wt)	Baicalin ($\mu g \cdot mg^{-1}$, dry wt)	Wogonin ($\mu g \cdot mg^{-1}$, dry wt)
1 μmol/L GA_3	1.0476 bc	0.000 e	—	—	—
MSO	2.8571 b	0.0950 d	0.146 i	4.00 f	0.071 g
2.5 μmol/L IAA	5.1429 a	15.381 a	1.580 b	12.7 b	0.286 b
2.5 μmol/L IAA[a]/MSO	4.6667 a	10.619 b	0.489 e	3.55 g	0.087 e
2.5 μmol/L IAA[b]/1 μmol/L GA_3	2.4286 b	5.4760 c	1.250 c	11.1 c	0.209 c
2.5 μmol/L IAA[b]/5 μmol/L GA_3	2.1429 bc	5.9050 c	2.700 a	14.9 a	0.540 a
2.5 μmol/L IAA[b]/10 μmol/L GA_3	1.6667 bc	4.0480cd	0.525 d	5.48 d	0.102 d
2.5 μmol/L IAA[b]/20 μmol/L GA_3	1.6190 bc	4.2380 cd	0.218 h	4.00 f	0.041 i
2.5 μmol/L IAA +1 μmol/L GA_3[c]/MSO	1.9048 bc	5.0000 cd	0.364 f	4.17 f	0.069 g
2.5 μmol/L IAA +5 μmol/L GA_3[c]/MSO	1.0952 bc	4.2380 cd	0.313 g	5.29 e	0.062 h
2.5 μmol/L IAA +10 μmol/L GA_3[c]/MSO	1.000 bc	2.6670 cd	0.280 g	3.97 f	0.077 f
2.5 μmol/L IAA +20 μmol/L GA_3[c]/MSO	0.4286 c	0.7620 cd	0.293 g	5.64 d	0.061 h

1 μmol/L GA_3: Explants were cultured on 1 μmol/L GA_3 medium for 30 days; 2.5 IAA: Explants were cultured on 2.5 μmol/L IAA medium for 30 days; MSO: Explants were cultured on MSO medium for 30 days. [a] Explants were pre-cultured on 2.5 μmol/L IAA medium for 6 days before transferred to MSO medium. [b] Explants were pre-cultured on 2.5 μmol/L IAA medium for 6 days before transferred to MSO medium containing GA_3 at the concentrations of 1,5,10 and 20 μmol/L. [c] Explants were pre-cultured on 2.5 μmol/L IAA medium containing GA_3 at the concentrations of 1,5,10 and 20 μmol/L for 6 days before transferred to MSO medium. Data recorded after 30 days of culture.

2.3 Effect of GA_3 on flavonoid content

Ultimately, Huang-qin tissues are grown for their capacity to produce medicinal metabolites. Analyses of the concentration of bioactive compounds in the explants demonstrated that the addition of GA_3 to 2.5 μmol/L TDZ or 2.5 μmol/L IAA for 6 days or MSO media for 24 days had different influences on the concentration of bioactive compounds (Table 1, 2). In IAA-treated control, IAA significantly promoted their concentrations in the explants in continuous darkness. The concentrations of baicalein, baicalin and wogonin have about 10-, 3- and 4-fold increase in compared to MSO control, respectively. However, GA_3 added to 2.5 μmol/L IAA medium had a negative influence on the concentrations of the three compounds (Table 2). The increased concentrations of the three compounds were seen only at 1-5 μmol/L GA_3 added to MSO medium. Maximum enhancement was observed at 5

μmol/L to about 5.5-, 4.2- and 6.2-fold of the control, respectively, which was higher than those of IAA-treated control. The highest concentrations of baicalein, baicalin and wogonin in continuous darkness were at 5 μmol/L GA_3 added to MSO medium with dry weights of 2.70, 14.90 and 0.54 $\mu g \cdot mg^{-1}$, respectively (Table 2).

In TDZ-treated control, TDZ had no effect on the concentrations of baicalein and wogonin in comparison with MSO-control, but increased the concentration of baicalin to about 2-fold of the MSO control. The concentrations of wogonin in all GA_3 treatments were trace. In the control, TDZ reduced the concentrations of baicalein and wogonin but enhanced the concentration of baicalin to about 3.5-fold of MSO control. The addition of GA_3 to 2.5 μmol/L TDZ medium had less significant influence on the concentrations of the baicalein and wogonin, but decreased concentration of baicalin in comparison with the control. The addition of GA_3 to MSO medium decreased the concentrations of baicalein and baicalin in comparison with the control (Table 1).

One of the major limitations to the development of high-quality plant-based medicines is the need to adapt both traditional and high-tech agricultural practices to unusual species. Yield of medicinal plants needs to be defined both in biomass, chemical composition and the quality. Our results show that there is a great potential of using exogenous auxin for the promotion of pharmacological active compounds of Huang-qin. GA_3 use at low concentration is expected to be effective, when explants are cultured on 2.5 μmol/L IAA-treated induction medium for 6 days and then cultured on MSO medium supplemented with 1-5 μmol/L GA_3 for 24 days. Under the conditions, maximum concentrations of baicalein, baicalin and wogonin in the explants of HQ_{111} were obtained in comparison with those reported previously (Murch *et al.*, 2004; Zeng *et al.*, 2007). Therefore, interaction between exogenous GA_3 and IAA should be further investigated.

In conclusion, the present investigation implicated both the exogenous and endogenous GA_3 playing a stimulatory role during shoot development of Huang-qin germplasm line HQ_{111}. The endogenous content of GA_3 is probably not optimal for shoot formation. However, the level of endogenous gibberellin for induction and growth of root presumably is adequate. Tissue culture techniques can play an important role in clonal propagation of elite genotypes of Huang-qin.

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赤霉素 GA_3 调节黄芩组织培养中芽和根的形成

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摘 要：应用组织培养技术对黄芩进行外源激素调控研究。在培养不同时间进行的不同培养基之间的转移培养研究表明，组织培养条件下，在培养基中添加赤霉素，可显著刺激黄芩外植体芽的形成，同时抑制根的生长。在加有 GA_3 的 IAA 培养基上，GA_3 显著影响黄芩组织培养物中的黄酮含量。在黑暗条件下，开始在 2.5 μmol/L IAA 培养基中培养 6 d，随后转移到 5 μmol/L GA_3 培养基上培养，黄芩外植体中黄岑苷、黄岑素和汉黄芩苷的含量最高，分别为 14.90，2.70 和 0.54 μg mg^{-1}（干重）。

关键词：不定根；黄芩；赤霉素；黄岑苷；黄岑素；汉黄芩苷

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