ENDOGENOUS HORMONE CONCENTRATIONS IN EXPLANTS AND CALLUSES OF BITTER MELON (*Momordica charantia* L.)

Y. Tang, J. Liu, B. Liu, X.M. Li, J. Li and H.X. Li

SUMMARY

*Anthers and stems of bitter melon (*Momordica charantia *L.) cv Bixiu were used for in vitro culture establishment. The endogenous hormone concentrations (indoleacetic acid (IAA), abscisic acid (ABA), gibberellins 3 (GA_3), and zeatin (ZT)) of the initial explants and calluses were determined by means of high pressure liquid chromatography (HPLC). The endogenous IAA and IAA/ZT ratio were higher in the explants that were more effective to induce callus, and ABA was negative to callus formation. When analyzing the endogenous hormone concentrations in the various callus types generated in anthers and stems, it was found that a higher concentrations of ZT was present in the stem calluses that had formed buds, while higher IAA/ZT and GA_3/ZT ratios were present in the calluses having no bud formation capacity, originated from anthers and stems.*

CONCENTRACIONES DE HORMONAS ENDÓGENAS EN EXPLANTES Y CALLOS DE MELÓN AMARGO (*Momordica charantia* L.)

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RESUMEN

*Se utilizaron anteras y tallos de león amargo (*Momordica charantia *L.) cv Bixiu para establecer un cultivo in vitro. Las concentraciones de hormonas endógenas (ácido indolacético (IAA), ácidso abcísico (ABA), giberelinas (GA3) y zeatina (ZT)) en los explantes y callos iniciales fueron determinadas por medio de cromatografía líquida de alta presión (HPLC). El IAA endógeno y la razón IAA/ZT fueron mayores en los explantes que fueron más efectivos en inducir la formación de callos, mientras que el ABA impidió su formación. Al analizar las concentraciones de hormonas endógenas en los diversos tipos de callo generados de anteras y tallos se observó que una mayor concentración de ZT estuvo presente en los callos de tallos que habían formado yemas, mientras que en los callos sin capacidad de formación de yemas, originados de anteras y tallos, las relaciones IAA/ZT y GA3/ZT fueron mayores*

Introduction

Bitter melon (*Momordica charantia* L.) is one of the most nutritional and medicinal plants belonging to the Cucurbitaceae family. It contains high concentrations of ascorbic acid and iron (Behera *et al*., 2008). Bitter melon has been used as a traditional medicine for diabetes in India, China, and Central America (Grover *et al*., 2002; Yeh *et al*., 2003). It has been found that this vegetable possesses effective components in preventing HIV (Lee-Huang *et al*., 1990, 1995).

Anther culture is a useful tool for the rapid generation of haploid plants for use in plant breeding programmes (Massiah *et al*., 2001); however, there are few reports on anther culture in bitter melon. After three years concentrated on it, it was found that it is easy to induce calluses and very difficult to differentiate buds (Tang *et al*., 2009). Simultaneously, a similar phenomenon was found for *in vitro* propagation from stems.

Endogenous hormone levels have been regarded as critical to callus and bud formation, and even plant regeneration at *in vitro* culture for many plant species (Hiroshi *et al*., 1991; Martínez and Halac, 1995; Centeno *et al*., 1996; Valdés *et al*., 2001; Zhang *et al*., 2008). No report was found on endogenous hormones of explants and calluses during *in vitro* culture in bitter melon. A better understanding of the relationship between endogenous hormone concentrations in the original explants and the calluses, and their competence will help to achieve anther culture and *in vitro* propagation in bitter melon. In the present work, endogenous hormones on explants and calluses were measured and correlated with their ability to grow in culture.

KEYWORDS / Bitter Melon / Endogenous Phytohormones / HPLC / *In Vitro* Culture / *Momordica charantia* L. /

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CONCENTRAÇÕES DE HORMONAS ENDÔGENAS EM EXPLANTES E CALOS DE MELÃO-DE-SÃO-CAETANO (*Momordica charantia* L.)

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RESUMO

*Utilizaram-se anteras e caules de melão-de-são-caetano (*Momordica charantia *L.) cv Bixiu para estabelecer um cultivo in vitro. As concentrações de hormonas endôgenas (ácido indolacético (IAA), ácido abcísico (ABA), giberelinas (GA_3) e zeatina (ZT)) nos explantes e calos iniciais foram determinadas por meio de cromatografia líquida de alta pressão (HPLC). O IAA endôgeno e a razão IAA/ZT foram maiores nos explantes que foram mais efetivos em induzir a formação de calos, enquanto que o ABA impidiu sua formação. Ao analisar as concentrações de hormonas endôgenas nos diversos tipos de calo gerados de anteras e caules se observou que uma maior concentração de ZT esteve presente nos calos de caule que haviam formado gemas, enquanto que nos callos sem capacidade de formação de gemas, originados de anteras e caules, as relações IAA/ZT e GA_3/ZT foram maiores*

Materials and Methods

Plant material

Young viridescence flower buds ~5mm long and tender stems ~2mm in diameter were collected from bitter melon cv Bixiu plants grown in experimental plots using standard agronomic practices. Flower buds and stems were surface-sterilized with 75% (v/v) alcohol for 1min, then immersed in 0.1% (w/v) mercuric chloride solution with periodic agitation for 5min, and finally washed five times with sterile distilled water. The intact anthers after filament elimination and stems divided in 10mm-long segments were inoculated on MS medium (Murashige and Skoog, 1962) containing 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5mg·l^{-1} and benzyladenine (BA) 2.0mg·l^{-1}. After 20 days the explants that developed calluses were transferred to a subculture medium consisting of MS mineral salts and vitamins, thidiazuron (TDZ; 0.05, 0.1, 0.5 and 1mg·l^{-1}) in combination with 2,4-D (0.1, 0.5 and 1mg·l^{-1}). Subsequent subcultures were carried out every 20 days. All culture media were supplemented with 3% (w/v) sucrose, 0.7% (w/v) and agar, and the pH was adjusted to 5.8 before autoclaving. Cultures were maintained in growth chambers at 25ºC in the dark for 5 days, and then at 25°C under 16h daily illumination with 1500lx fluorescent light.

Hormone concentration of anthers and stems

To determine the possible influence of the endogenous hormonal status of explants, anthers and stems were excised as previously described for the *in vitro* culture and the endogenous hormone concentrations analyzed as outlined below.

Hormone concentration of calluses

After 60 days of culture under the aforementioned maintenance conditions, samplings were carried out to evaluate differences in the endogenous hormone concentrations of the calluses.

Determination of hormone levels

Instruments and reagents. A Varian Pro STAR 240 high performance liquid chromatograph and a Milli-Q ultrapure water purification system were used. Standards of abscisic acid (ABA), indoleacetic acid (IAA), gibberellins 3 (GA_3) and zeatin (ZT) were from Sigma Chemical, chromatographic purity methanol from Fisher Chemical, ethanoic acid was analytical grade, and the water used in the experiment was ultrapure water.

Chromatography. A Hypersil ODS C18 chromatographic column (150×4.6mm, 5μm) was used, employing as mobile phase a mixture of methanol and 0.6% ethanoic acid. Gradient elution was applied as follows: 5%-75% methanol from 0 to 13min, and 75% methanol from 13 to 15min. Column temperature was 35ºC, sample size 10μl, flow rate 1ml·min^{-1}, and UV detection wavelength of 254nm.

Sample treatment and determination of hormone levels. Determinations of IAA, ABA, GA_3 and ZT were performed on the same sample. Anther samples collected were surface dried and cleaned with a paper towel, immediately weighed and frozen in liquid nitrogen and stored at -70ºC. Samples (~1g fresh weight; FW) were ground in liquid nitrogen, homogenized and then extracted overnight with 30ml of 80% cold aqueous methanol (<0ºC) in darkness at 4ºC. The extract was centrifuged at 5000rpm and 4ºC for 15min and the supernatant was collected. Then, fresh cold methanol was poured onto the remnant, extracted three times as stated above. The total methanolic extract was dried in a rotary evaporator and dissolved in 10ml methanol. IAA, ABA, GA_3 and ZT were measured by injection of the extract into a reverse-phase HPLC.

Statistical analysis

A randomized complete block design was used. For callus induction, 10 explants per conical flask were inoculated in 100ml flasks containing 30ml of nutrient medium each, with 30 replicates per treatment. For differentiation, each treatment was applied to 30 calluses (5 calluses per conical flask and 6 replicates per treatment). Endogenous hormonal concentrations were determined in at least three biological replications. Significance between means was tested by Duncan's multiple range test (Duncan, 1955).

Results

Comparative analysis of callus response

After 7 days in culture, the anther and stem explants expanded and showed evidence of swelling at the cut edge. The calluses increased in size along the time of culture. After being cultured for 20 days, 89.3% stems and 62.7% anthers had induced calluses.

After being transferred to the subculture medium, the calluses originated from the stems and subcultured in two types of media (MS medium containing 0.1mg·l^{-1} 2,4-D and either 0.5 or 1mg·l^{-1} TDZ) proliferated, turned into a green color and showed few green protuberances (Figure 1a). At the third subculture, buds emerged from the surface of these protuberances (Figure 1b). On the contrary, calluses of stems subcultured in the other media employed

just proliferated and lacked any sign of organization, and even some of them showed browning (Figure 1c, d). In contrast, most of the calluses induced from anthers turned soft and translucent (Figure 1e), while others turned into brown (Figure 1f), and bud formation did not occur in these calluses. In short, there was no bud formation from the anther calluses subcultured in all 12 types of media, while a few buds differentiated from some of the stem calluses subcultured in the two media specified before. The bud formation rate was only 5.6%.

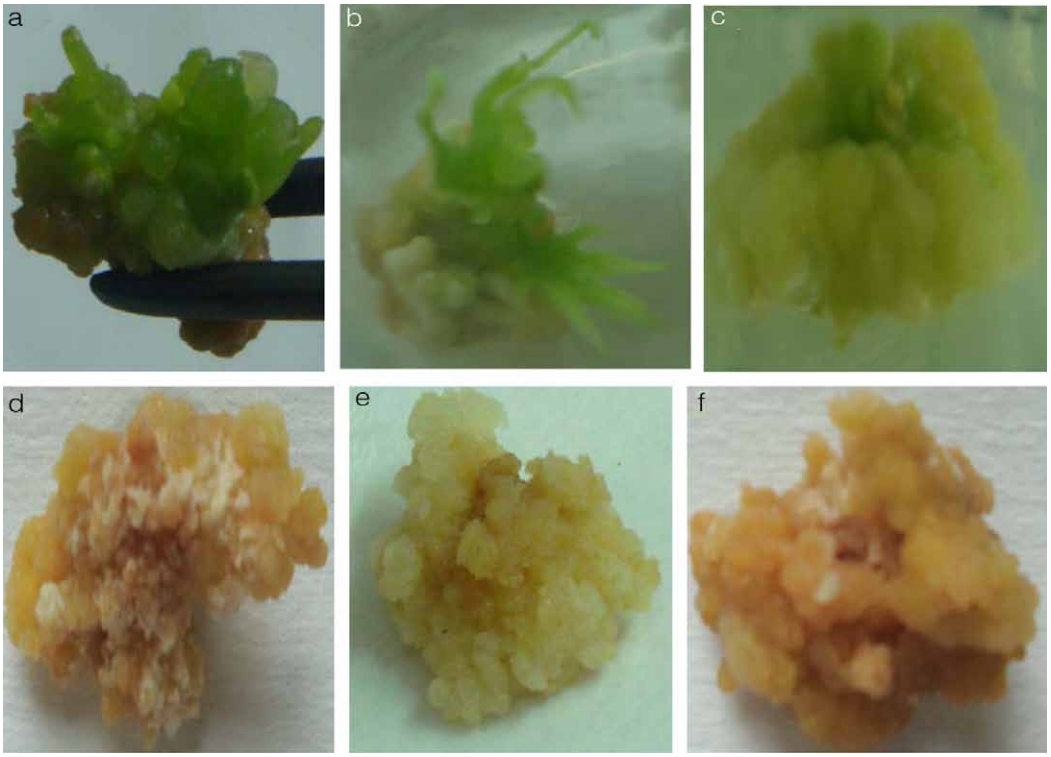

Figure 1. Calluses originated from stems and anthers in bitter melon. a: stem callus with green protuberances, b: adventitious buds regenerated from the surface of protuberances, c: stem callus proliferation without any sign of organization, d: browning stem callus. e: soft and translucent anther callus, and f: browning anther callus.

Hormone concentration of anthers and stems

The endogenous hormone concentrations in anthers and stems are presented in Figure 2. The ZT concentrations in the anthers were two times higher than those in the stems. However, higher concentrations of IAA were found in stems as compared to those of anthers. No statistically significant difference was found in the concentration of GA_3 between anthers and stems, and both were comparatively high. Anthers contained higher concentrations of ABA than stems. With respect to the IAA/ZT and GA_3/ZT ratios, they were significantly higher in stems than in anthers.

Hormone concentration of calluses

In the subsequent hormone analysis, anther calluses in four types of media and stem calluses differentiated no buds in two types of media out of the 12 types of media randomly selected, and stem calluses differentiated buds in the two media. The endogenous hormone concentrations in the calluses of the eight categories mentioned above are presented in Figure 3. Higher concentrations of ZT were found in the calluses that had formed buds (B) of stems as compared to the concentrations measured in the other callus types, both in the calluses that had no bud formation capacity (NB) generated from stem and anther. No differences were found in the IAA concentrations between the different callus categories. Calluses originating from stems contained lower concentrations of GA_3 and ABA than those originating from anthers, and the concentrations of stem B calluses were slightly lower than those from stem NB calluses. The IAA/ZT ratio was significantly lower in the B calluses than in the other callus types, and the same phenomenon was observed with respect to the GA_3/ZT ratio.

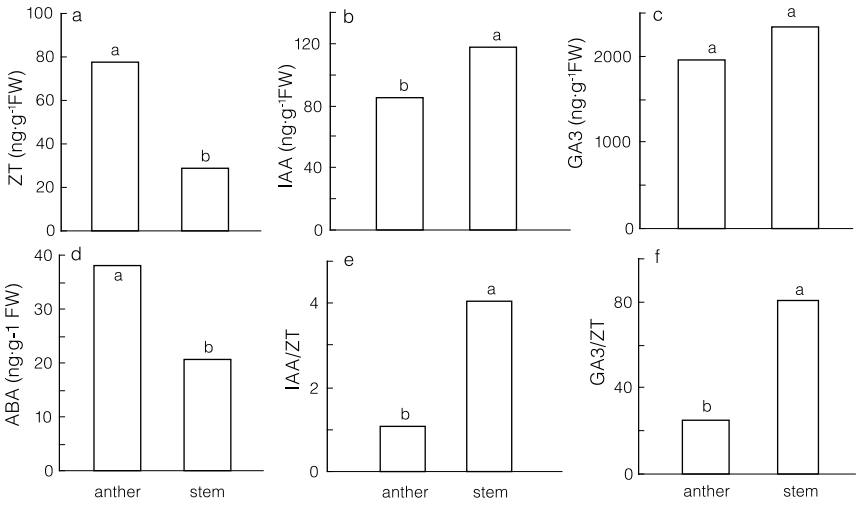

Figure 2. Endogenous concentrations of ZT (a), IAA (b), GA_3 (c), ABA (d), IAA/ZT ratio (e) and GA_3/ZT ratio (f) in anthers and stems of bitter melon. Significant differences (<0.05) between the concentrations and ratios of anther and stem are indicated by with distinct letters.

Discussion and Conclusions

In the present study, it was found that the callus formation rate of stems (89.3%) was higher than that of anthers (62.7%), although both were comparatively high. According to these results and other reports (Centeno *et al*., 1996; Valdés *et al*., 2001), endogenous IAA concentrations may play an important role in callus formation, and the explants with higher contents of IAA were easier to induce callus formation. In the present study, high ABA content was negative to callus

induction, when the endogenous ABA concentrations in the anther and stem were compared. As previously stated, IAA/ZT and GA_3/ZT ratios were significantly higher in stems than in anthers. In this sense, a positive influence of the IAA/ZT ratio for callus formation was found by Branca *et al.* (1991).

The analysis of the endogenous hormone concentrations of the different types of bitter melon callus showed that the major differences observed were a higher concentration of ZT in the B calluses from stems, and higher IAA/ZT and GA_3/ZT ratios in the NB calluses. In the present work, significantly higher ZT concentrations were found in the calluses that formed buds, and inclusion of the ZT in this study was consistent with the earlier reports (Yoshimatsu and Shimomura, 1994; Sarul *et al*., 1995). Kopertekh and Butenko (1995) found high ABA concentrations in the genotypes that were easier to regenerate; the present results differ from theirs, since lower ABA concentrations were found in the stem B calluses than in the stem NB calluses. This may be because different genotypes were evaluated in both studies. In the current study, a lower IAA/ ZT ratio in the stem calluses seemed to be involved in the presence of bud formation, in agreement with results in banana (Zaffari *et al*., 2000). The situation of GA_3 was less clear and reports showed ambiguous data (reviewed by Jiménez, 2005). With respect to the GA_3 in stem calluses, the high concentration may suppress adventitious bud formation, and a lower GA_3/ ZT ratio in stem calluses was considered as a crucial factor to differentiate buds.

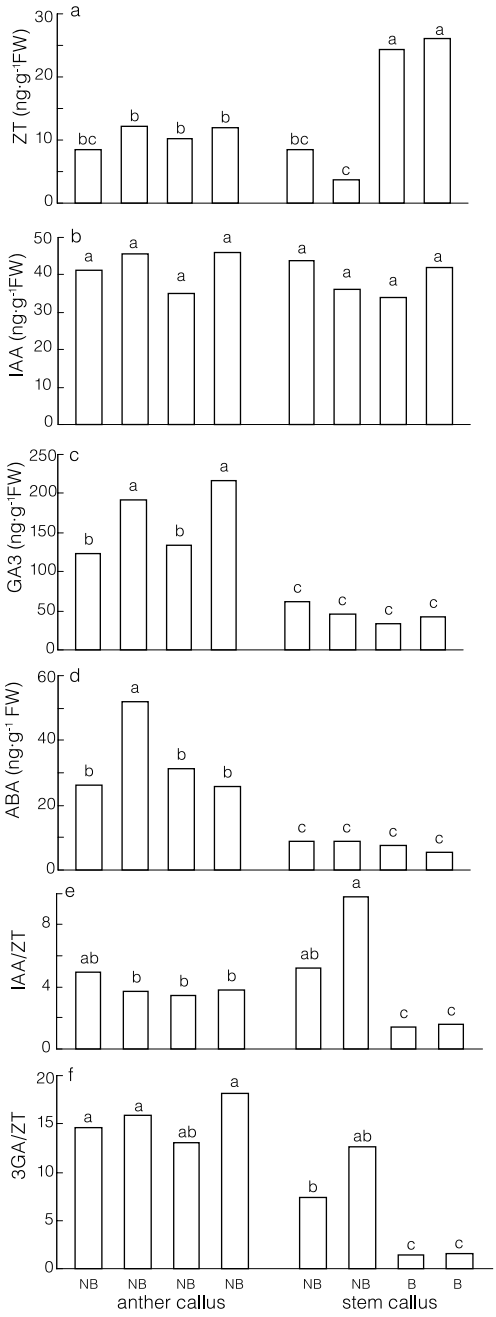

Figure 3. Endogenous concentrations of ZT (A), IAA (B), GA_3 (C), ABA (D), IAA/ZT ratio (E) and GA_3/ZT ratio (F) in the different bitter melon callus categories. B: calluses that had formed buds. NB: calluses that had no bud formation capacity. Significant differences (<0.05) between the concentrations and ratios of anther and stem are marked with distinct letters.

To the best of our knowledge, this is the first work in which anthers, stems and different types of calluses in bitter melon have been analyzed separately regarding their endogenous hormone concentrations.

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