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Efficient plant regeneration in vitro from cotyledon explants of chieh-qua (*Benincasa hispida* Cogn. var. chieh-qua)

E.E. Liu[a], David W.M. Leung[b], Qing Hua Xia[a], Jie Ren Zheng[a], Xin Xiang Peng[a], Xiao Ming He[c]

[a] College of Life Science, South China Agricultural University, Guangzhou 510642, China
[b] School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand
[c] Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

*Corresponding author, e-mail: xiaominghe@tom.com

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ABSTRACT: An efficient plant regeneration system for *Benincasa hispida* Cogn. var. chieh-qua was developed using explants isolated from mature seeds germinated on moist filter paper under aseptic conditions for 3 days in the dark and 2 days under light. Shoot regeneration frequency (percentage of explant-forming shoots) was about 90% when the proximal cotyledon portion with a 1 mm-long hypocotyl segment was first cultured on Murashige and Skoog (MS) medium supplemented with 6.0 mg/l benzylaminopurine (BA) and 0.2 mg/l naphthalene acetic acid (NAA) for 4 days and then transferred to MS medium containing 1 mg/l BA for 4 weeks. About 80% of the regenerated shoots could elongate further. The root induction medium was half-strength MS medium with 0.01 mg/l NAA and 0.01 mg/l BA. The average rooting frequency was about 93%.

KEYWORDS: adventitious shoot formation, organogenesis, pre-culture, hairy melon

INTRODUCTION

Chieh-qua (*Benincasa hispida* Cogn. var. chieh-qua How, hairy melon) is a primary vegetable crop in South China and is a member of the Cucurbitaceae crops. In recent years, as a result of the ever expanding and more intense cropping, there is an increasing threat of more frequent outbreaks of diseases which would severely curtail the hairy melon supply. Therefore, there is a great urgency to develop new disease-resistant chieh-qua germplasm. One effective approach to solve this problem is the use of genetic engineering to transfer heterologous disease-resistance genes into existing germplasm of chieh-qua. An important prerequisite for a successful plant transformation is the development of a yet unavailable efficient plant regeneration system in vitro for chieh-qua[1].

Up to now, there have been a number of studies on in vitro regeneration of several Cucurbitaceae crops including cucumber[2,3], watermelon[4,5], melon[6], squash[7,8], bottle gourd[9], figleaf gourd[10], ash gourd[11], muskmelon[12], and chieh-qua[1]. However, only in cucumber, watermelon, squash, and melon, efficient and reliable plant regeneration protocols have been established. The regeneration system of chieh-qua is still problematic because the frequency of shoot regeneration was low and unstable (the highest was 68%), and moreover, the adventitious shoots formed were stunted and difficult to elongate, in spite of the effects of photoperiod, genotype, medium type, and various plant growth regulators on explants regeneration had been assayed[13]. Therefore, the present study aimed to optimize regeneration of chieh-qua by analysing the effects of plant growth regulators, pre-culture treatments, and different explant types.

MATERIALS AND METHODS

Seed germination under aseptic conditions

Seeds of an inbred line of chieh-qua called A39 provided by Guangdong Province Agricultural Institute, China, were used. Seed coats were removed and then disinfected with 5% (v/v) sodium hypochlorite for 15 min followed by five rinses with sterile distilled water. Then, about 20 disinfected seeds were placed on a paper bridge dipped in sterile deionized water in an Erlenmeyer flask. A growth room kept at 25 °C with the following illumination conditions was used for seed germination: 3 days in the dark followed by 2 days of a 12/12 h photoperiod with irradiance of 40 μmol m^{-2} s^{-1}.

Plant regeneration experiments

Five types of explants were prepared for investigation of their response to the induction of shoot regeneration

in vitro. Firstly, the root of an aseptically grown chieh-qua seedling was excised and discarded. Then the cotyledons were cut in the middle into the distal and proximal halves with reference to the point at which the hypocotyl was attached to the cotyledons. The distal cotyledon halves were discarded. The hypocotyl was split longitudinally into two halves each still attached to a proximal cotyledon half. A type 1 explant was a proximal cotyledon half attached to a small portion (about 3 mm) of the split hypocotyl. A type 2 explant was the junction region consisting of a small portion (about 1 mm) of the proximal cotyledon half still attached to a small portion (about 3 mm) of the hypocotyl half. A type 3 explant was the proximal cotyledon half attached to 1 mm-long of the split hypocotyl. Type 4 explants consisted of only the proximal cotyledon halves. Type 5 explants were similar to the type 4 explants except that the 2 mm-long cotyledon segment closest to where the hypocotyl half was attached was excised and discarded. The explants were placed with the adaxial side down on basal Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose, 6 mg/l benzylaminopurine (BA) and 0.2 mg/l naphthalene acetic acid (NAA) for 4 days before they were transferred to MS medium supplemented with 1 mg/l BA and 0.2 mg/l NAA. After 4 weeks of culture, the percentages of explants producing shoots were determined.

To investigate the effect of different concentrations of BA on shoot regeneration, type 3 explants were placed with the adaxial side down on basal MS medium supplemented with 3% (w/v) sucrose and different concentrations (1, 2, 4, or 6 mg/l) of BA in combination with 0.2 mg/l NAA. To investigate the influence of pre-culture treatments, type 3 explants were pre-cultured on MS medium supplemented with 6 mg/l BA and 0.2 mg/l NAA for 2, 3, 4, and 5 days, respectively, before they were transferred onto media supplemented with 1, 2, or 4 mg/l BA and 0.2 mg/l NAA. In a follow-up experiment, type 3 explants were pre-cultured for 4 days on medium supplemented with 6 mg/l BA and 0.2 mg/l NAA before transferred to media supplemented with 1 mg/l BA and 0, 0.1, or 0.2 mg/l NAA. After 4 weeks of culture, the percentages of explants producing shoots (herewith referred to as regeneration frequency in percent) in each treatment were determined.

Elongated shoots were excised and rooted on half-strength MS medium solidified with 3 g/l phytagel, supplemented with 0.01 mg/l BA alone or in combination with 0.01 mg/l NAA or 0.05 mg/l indole-3-acetic acid (IAA). The number of roots formed per cultured shoot (root/shoot ratio), percentage of shoots forming roots (rooting frequency) and root length were determined after about 2 weeks of culture. The pH of all the media was adjusted to 5.8 prior to the addition of agar (1% w/v) or phytagel as required. All media were autoclaved at 121°C for 15 min before use. There were three replicates (60 explants) in each treatment. All cultures were kept in a growth room at 25°C and under a 12/12 h photoperiod with irradiance of 40 µmol m^{-2} s^{-1}.

Table 1 Effect of explant type on shoot regeneration frequency (percentage of explants forming shoots).

Ex. type	Description of explant	Reg. (%)*
1	Proximal cotyledon portion with hypocotyl segment	87 ± 2^{a}
2	Junction of hypocotyl and cotyledon	82 ± 2^{b}
3	Proximal cotyledon portion with 1 mm-long hypocotyl segment	86 ± 1^{a}
4	Proximal cotyledon portion	38 ± 2^{c}
5	Proximal cotyledon portion with the most proximal 2 mm removed	0^{d}

* Values of regeneration followed by a different letter are statistically different at 5% level.

Data analysis

For each treatment, data were statistically analysed using a spreadsheet application, and significant differences between various treatments were analysed at 5% level by Duncan's new multiple range method of DPS v6.55 (DPS Soft Inc., Tang, Hangzhou, China.) analytical software.

RESULTS

Effect of type of cotyledon explants on shoot regeneration

Among the five types of explants tested for inducing shoot regeneration, only type 5 explants did not form any shoots. Shoot regeneration frequency was slightly lower in type 2 explants than in type 1 explants. Nevertheless, a majority (more than 80%) of type 1 to type 3 explants formed shoots. In contrast, only 38% of type 4 explants regenerated shoots (Table 1).

Effect of BA on shoot regeneration

Type 3 explants (proximal cotyledon half attached to 1 mm-long split hypocotyl segment) were cultured on MS medium containing different concentrations of BA in combination with 0.2 mg/l NAA for 4 weeks. The percentage of the explants forming shoots was significantly higher on the medium containing 6 mg/l BA than 1 mg/l BA. There was no significant difference in the percentage of explants forming shoots on

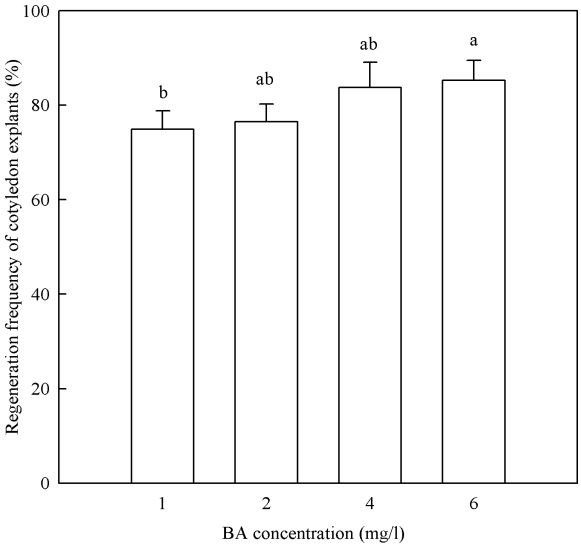

Fig. 1 Effect of different BA concentrations with 0.2 mg/l NAA on shoot regeneration frequency (percentage of explants forming shoots) in type 3 cotyledon explants of chieh-qua. Data were obtained after 4 weeks of culture.

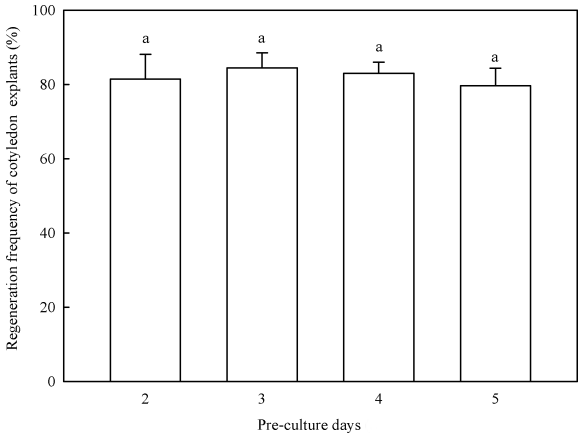

Fig. 2 Effect of pre-culture time on shoot regeneration frequency (percentage of explants forming shoots) in type 3 explants cultured on medium with 6 mg/l BA and 0.2 mg/l NAA. Data were obtained after transfer to followed medium with 1 mg/l BA and 0.2 mg/l NAA for 4 weeks.

media containing 2, 4, or 6 mg/l BA (Fig. 1). However, the shoots formed seemed to elongate slightly.

Effect of pre-culture time and transfer to media with varying BA or NAA concentrations

No obvious effect of the pre-culturing treatments was found as far as the percentage of explants forming shoots after 4 weeks of transfer to medium with 1 mg/l BA and 0.2 mg/l NAA (Fig. 2). However, the shoots in the explants cultured on medium supplemented with 1 mg/l BA appeared to elongate more than those in the explants cultured with 2 or 4 mg/l BA. Following pre-culture for 4 days, shoot regeneration frequency could reach 90% when the explants were cultured on MS medium supplemented with 1 mg/l BA alone (Fig. 3). The presence of NAA (0.1 or 0.2 mg/l) in addition to BA decreased shoot regeneration frequency to about 80%.

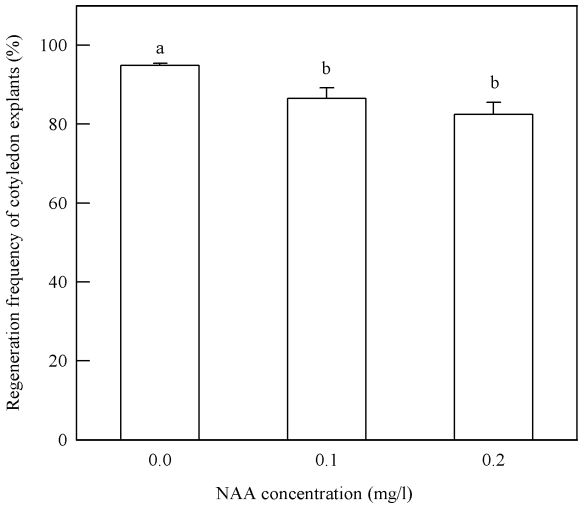

Fig. 3 Shoot regeneration frequency (percentage of explants forming shoots) in type 3 chieh-qua cotyledon explants after transfer to culture media containing 1 mg/l BA and different NAA concentrations. The explants were pre-cultured for 4 days on the medium containing 6 mg/l BA and 0.2 mg/l NAA before transferred. Data were obtained after 4 weeks of culture.

Effect of plant growth regulators on in vitro rooting of regenerated shoots

After 4 weeks of culture, well-developed shoots obtained by pre-culturing explants on the MS media supplemented with 6 mg/l BA and 0.2 mg/l NAA for 4 days before they were transferred onto MS medium supplemented with 1 mg/l BA were excised and rooted. There was no significant difference in rooting frequency (percentage of explants forming roots) whether the shoots were cultured on half-strength MS medium supplemented with 0.01 mg/l BA alone or in combination either with 0.05 mg/l IAA or 0.01 mg/l NAA. But shoots cultured on the medium supplemented with BA alone produced fewer roots per shoot than those cultured on the BA-containing media supplemented with 0.05 mg/l IAA or 0.01 mg/l NAA. Maximum rooting (100%) as well as production of

Table 2 Effect of plant growth regulators on rooting of in vitro regenerated shoots.

Culture medium (1/2 MS +)	Rooting frequency (%)*	Root/shoot ratio	Root length (cm)
0.01 mg/l BA	93.9 ± 5.4^{a}	4.7 ± 1.9^{b}	3.6 ± 1.9^{a}
0.01 mg/l BA + 0.01 mg/l NAA	93.5 ± 5.8^{a}	7.1 ± 3.1^{a}	3.3 ± 1.5^{ab}
0.01 mg/l BA + 0.05 mg/l IAA	100.0 ± 0.0^{a}	8.0 ± 3.0^{a}	3.1 ± 1.7^{b}

* Values of rooting frequency, root/shoot ratio, and root length followed by a different letter, in a row, are statistically different at 5% level.

normal roots (8 roots per shoot) were observed in shoots cultured on the 1/2 MS medium supplemented with 0.01 mg/l BA and 0.05 mg/l IAA. However, there was no significant difference in the number of roots per shoot, root length and rooting frequency of shoots cultured in the media supplemented with IAA and NAA (Table 2).

DISCUSSION

The type of explant is an important factor for morphogenesis in tissue culture[6]. Preparation and position of the cotyledon explant in relation to other seed parts have a significant effect on the frequency in vitro shoot regeneration in Cucurbitaceae crops. In winter squash, only the cells in the proximal portion of the cotyledon have the potential for adventitious shoot formation[8]. In the present study, the frequency of shoot regeneration was reduced markedly after removing the hypocotyl from the cotyledon and removing the most proximal 2 mm of the cotyledon resulted in 0% shoot formation. Likewise, explants with the most proximal 2 mm of the cotyledons removed led to the loss of shoot regeneration in squash[7]. In pumpkin, cotyledonary explants without the proximal region also failed to regenerate shoots[14]. Similar observations in figleaf gourd[10], bottle gourd[9], and soybean[15] are consistent with the suggestion that the junction of the hypocotyl and proximal half of cotyledon was a highly regenerative region and crucial for adventitious shoot regeneration. These may be related to the differences in endogenous conditions prevalent in different seed parts, especially the level and composition of hormones. Endogenous cytokinins and auxins played an important role in the initiation of meristematic proliferation centre and the subsequent bud primordial dormancy in *Pinus pinea*[16]. The regeneration frequency of rice callus depended on the endogenous levels of abscisic acid and IAA[17]. The regeneration ability of the pumpkin cotyledon explants was also related to the endogenous isopentenyl adenosine content in cotyledonary explants[14]. In bottle gourd, $AgNO_3$ (an ethylene inhibitor) is able to promote shoot regeneration from proximal cotyledon explants[9].

Addition of cytokinins to culture media has been reported to be crucial for the induction of adventitious shoot and proliferation in vitro shoot regeneration of numerous Cucurbitaceae such as cucumber, bottle gourd, squashes, and a *Cucurbita* interspecific hybrid[3,7–9,18–20]. Cytokinin can be used alone or in combination with a low concentration of auxins[4,5,14]. In the present study, the shoot regeneration frequency increased with increasing concentrations of BA, but the adventitious shoots formed in media with high concentrations of BA were stunted in growth and elongated little. The buds regenerated from the cotyledonary nodes of common bean was also significantly enhanced as the concentration of BA increased but beyond the optimum 5 mg/l the shoot buds appeared to be developmentally suppressed and did not grow further[21]. Interestingly, most of the regenerated shoots could elongate and the regeneration frequency could reach 90% if the explants were pre-cultured on MS medium containing a combination of 6 mg/l BA and 0.2 mg/l NAA for 4 days before they were transferred onto MS medium supplemented with 1 mg/l BA without any auxin. This is consistent with another study on *Celosia* showing that it is important to investigate the influence of pre-culture and transfer of explants to media differing in plant growth regulator on elongation of in vitro-derived shoots[22]. In melon, the addition of NAA to subculture medium also reduced shoot regeneration frequencies[6]. The addition of gibberellic acid (GA) to elongation medium had no effects on elongation of adventitious shoots of chieh-qua (data not shown). This is in agreement with the results of Dong and Jia[4] on watermelon. In contrast, 99.2% of adventitious shoots of cucumber could elongate in medium supplemented with 4.44 μM BA and 1.44 μM GA[18]. Incorporation of GA and low levels of BA in the shoot elongation media could enhance shoot production of squash[7]. No significant difference was observed in the number of roots per shoot, root length, or rooting frequency of chieh-qua shoots cultured on 1/2 MS medium containing IAA or NAA. However, since NAA is more stable than IAA during culture and IAA has to be filter-sterilized before adding to autoclaved media, NAA is preferred for rooting of in vitro-derived roots of chieh-qua.

The protocol described here is a rapid and reliable in vitro plant regeneration system for chieh-qua via direct shoot organogenesis. The entire process, from

dry seeds to regenerated plantlets, only requires about two months. In addition, the morphological and physiological variation of shoots regenerated directly from explants could be largely minimized[23, 24]. Therefore, this simple and rapid in vitro plant regeneration procedure for chieh-qua is better than indirect adventitious shoot formation via callus formation which was difficult to achieve in chieh-qua. Our protocol can be incorporated into a gene transfer protocol, for example, *Agrobacterium tumefaciens*-mediated transformation of chieh-qua to develop improved disease-resistant plants.

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