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In Vitro Callogenesis and Regeneration of Cucumber Plants from Hypocotyl and Cotyledon

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ABSTRACT

In vitro culturing of cotyledon and hypocotyl can serve various purposes such as gene transfer and asexual reproduction. This study aimed to regenerate plants from cotyledon and hypocotyl explants of cucumber cultivars, the Iranian landrace Isfahan and Beta Alpha F1 hybrid. The experiment was carried out in the tissue culture laboratory of Azad University of Isfahan in 2023. It employed a factorial completely randomized design with two genotypes and 10 media in the three replicate dishes. The media consisted of MS supplemented with BAP (1, 2, 3, and 4 mg/l) combined with NAA (0.05, 0.1, 0.2, and 0.5 mg/l). The results showed that callus was successfully induced in both cotyledon and hypocotyl explants in both genotypes. The highest percentages of regeneration from cotyledons were observed in 3 mg/l BAP and 0.5 mg/l NAA and 4 mg/l BAP and 0.1 mg/l NAA, with 65% and 60%, respectively, for the Isfahan genotype and in 1 mg/l BAP and 0.2 mg/l NAA and 1 mg/l BAP and 0.5 mg/l NAA, both exhibiting a 60% regeneration rate for Beta Alpha. Furthermore, when Isfahani and Beta-alpha's hypocotyl explants were cultured in MS supplemented with 2 mg/l BAP and 0.1 mg/l NAA, and 1 mg/L BAP and 0.2 mg/L NAA had 30% and 55% rate of regeneration, respectively. The present findings indicate that induced callus or shoot regeneration depended on genotype and explants. Each genotype requires specific callogenesis and regenerated media to achieve desirable results. Overall, the results suggest that cotyledons in the Isfahan genotype and hypocotyls in the Beta-alpha variety exhibited better regeneration when tested with these plant growth regulators.

Keywords: *Cucumis sativus*, Tissue culture, Plant growth regulators, BAP, NAA

INTRODUCTION

Higher production, area harvested, and higher yield are evidence of the importance of cucumber (*Cucumis sativus* L.) in global agriculture (Asadi and Seguí-Simarro, 2021).

Cucumbers have a range of uses beyond just being a food item. Various parts of the cucumber plant, including its leaves, fruits, and seeds, have been found to possess therapeutic potential. This potential can be harnessed for applications such as cosmetics and wound healing. Researchers have even synthesized biomolecules loaded with metallic silver nanoparticles using cucumber leaves and callus tissues. They then studied the resulting nanodrug-based ointment for its wound-healing properties in a rat model. (Venkatachalam *et al*., 2015). An efficient protocol for high-frequency callus induction and plant regeneration systems could be a useful tool in cucumber for various purposes such as micropropagation, gene transfer, cell development studies, and more. Plant regeneration has been achieved via organogenesis in cucumber using different explants and different studies such as cotyledons (Gambley and Dodd, 1990, Selvaraj *et al*., 2007, Venkatachalam *et al*., 2018), hypocotyls (Selvaraj *et al*., 2006), shoot tips (Vasudevan *et al*., 2004), Somatic embryogenesis (Mashayekhi *et al*., 2012), Anther culture (Asadi *et al*., 2018, Gemes-Juhasz *et al*., 2002), Ovule culture (Gemes-Juhasz *et al*., 2002).

According to a study conducted by Mohiuddin *et al*. (1997) all tested cucumber cultivars showed poor shoot regeneration in proximal cotyledon and hypocotyl explants, as well as unresponsiveness for distal cotyledon and hypocotyl when subjected to AgNO3 in an invitro culture. Venkatachalam *et al*. (2018) conducted a study to investigate the effects of cytokinin, silver nitrate, and auxins on cotyledonary node explants. They found that 1.5 mg l−1 BAP was the optimal concentration for inducing a high frequency of multiple shoots. Interestingly, the highest percentage of multiple shoot regeneration and number of shoot buds were observed on MS medium containing 4.5 mg l−1 AgNO3 and 1.5 mg l−1 BAP. Song *et al*. (2019) investigate the effect of light and plant growth regulators on the growth of cucumber hypocotyl. The study conducted by Kim *et al*. (2000) investigated the impact of seedling age and hypocotyl length on the induction of adventitious shoots in cucumber seedlings. The researchers used excised hypocotyl segments with cotyledons on an MS medium containing 2.0 mg/L zeatin. The results showed that explants from 3- and 5-day-old seedlings with one cotyledon or two half-cotyledons, and short hypocotyl length (2mm) had a higher frequency of adventitious shoot induction. On the other hand, explants with long hypocotyls and two cotyledons produced fewer shoots. This research aims to optimize the micropropagation of cucumber through in vitro culture of both hypocotyl and cotyledon.

MATERIALS AND METHODS

Plant materials

The cucumber (*Cucumis sativus* L.) Beta Alpha F1 hybrid and the Iranian landrace Isfahan were used in this study. The seeds were surface sterilized with 70% ethanol for 30 s and then with 4 g/l sodium hypochlorite for 5 min, followed by three rinses in sterile water. Then, seeds were cultures on 0.8% agar in jar glasses under 16/8 photoperiod light/dark and controlled temperature of 25 ± 2 $^{\circ}$C at the growth chamber of the Plant Improvement and Seed

Production Center, Isfahan (Khorasgan) Branch, Azad University, Isfahan, Iran during the 2023.

Cotyledon and hypocotyl culture

After ten days of cultivation, the cotyledons were carefully separated from the stem connection point without any attachment to the bud. After that, they were sliced into small pieces measuring 5-6mm. Similarly, the hypocotyl samples were cut into pieces that were 4-5mm in size. The explants were plated in 100 * 15 mm petri dishes containing basal MS salts and vitamins supplements with growth regulators are detailed in Table 1, and then sub cultured every 14 days with freshly prepared medium until they produced calli and, or regeneration.

Statistical analysis

The experiment was laid in a factorial completely randomized design with two genotypes and 10 media in the three replicate dishes (10 explant/replicate). The percentage of callus induction and percentage of regeneration were measured. Test of significance was conducted using the SAS and Excel software and significant difference among treatments was calculated using Duncan's new multiple range test (LSR) Test at p 0.05.

RESULTS

Callogenesis

According to the data presented in Table 1, callus was successfully induced in both cotyledon and hypocotyl explants in the Beta Alpha F1 hybrid and the Iranian landrace Isfahan. The combination of different concentrations of NAA and BAP did not have a significant impact on callus induction. However, it was observed that the callus induced from hypocotyls explant was larger than the one induced from cotyledons (Figure 1a and Figure 2a). Moreover, balanced combinations of NAA and BAP were more effective for callus induction.

Regeneration

Table 1 presents the results of the direct and indirect regeneration of cotyledon and hypocotyl explants in Beta Alpha F1 hybrid and the Iranian landrace Isfahan, using various concentrations of NAA and BAP. The data obtained from the experiments showed that M8 (3 mg/l BAP and 0.5 mg/l NAA) and M9 (4 mg/l BAP and 0.1 mg/l NAA) had the highest percentages of regeneration from cotyledons, with 65% and 60%, respectively, for the Isfahan genotype. On the other hand, no plant regenerated on M2 (1 mg/l BAP and 0.1 mg/l NAA), M3 (1 mg/l BAP and 0.2 mg/l NAA), and M4 (1 mg/l BAP and 0.5 mg/l NAA) for this

genotype. It appears that low concentrations of BAP combined with high levels of NAA are unsuitable for cotyledon regeneration in the Isfahan genotype. The experiment results showed that when the concentration of BAP remained constant at 1 mg/l, but the concentration of NAA decreased to 0.05 mg/l, it resulted in budding (M1 media), on the other hand, an increase in BAP concentration to 2 mg/l (M5 and M6) led to plant regeneration. The regeneration of Beta alpha cotyledons was successfully induced in two of the tested mediums, M3 (1 mg/l BAP and 0.2 mg/l NAA) and M4 (1 mg/l BAP and 0.5 mg/l NAA), both of which exhibited a 60% regeneration rate. However, no significant response was observed in the other tested concentrations of BAP and NAA.

The results shown in Figure 1 indicate that both direct and indirect regeneration were observed in the tested media using cotyledon explant and calli derived from cotyledons, respectively. The regenerated plants were able to root and grow into normal plants when cultured in a 1.2 MS medium. These findings suggest that cotyledon culture could be a useful technique for micropropagation and research purposes, such as gene transfer in cucumber.

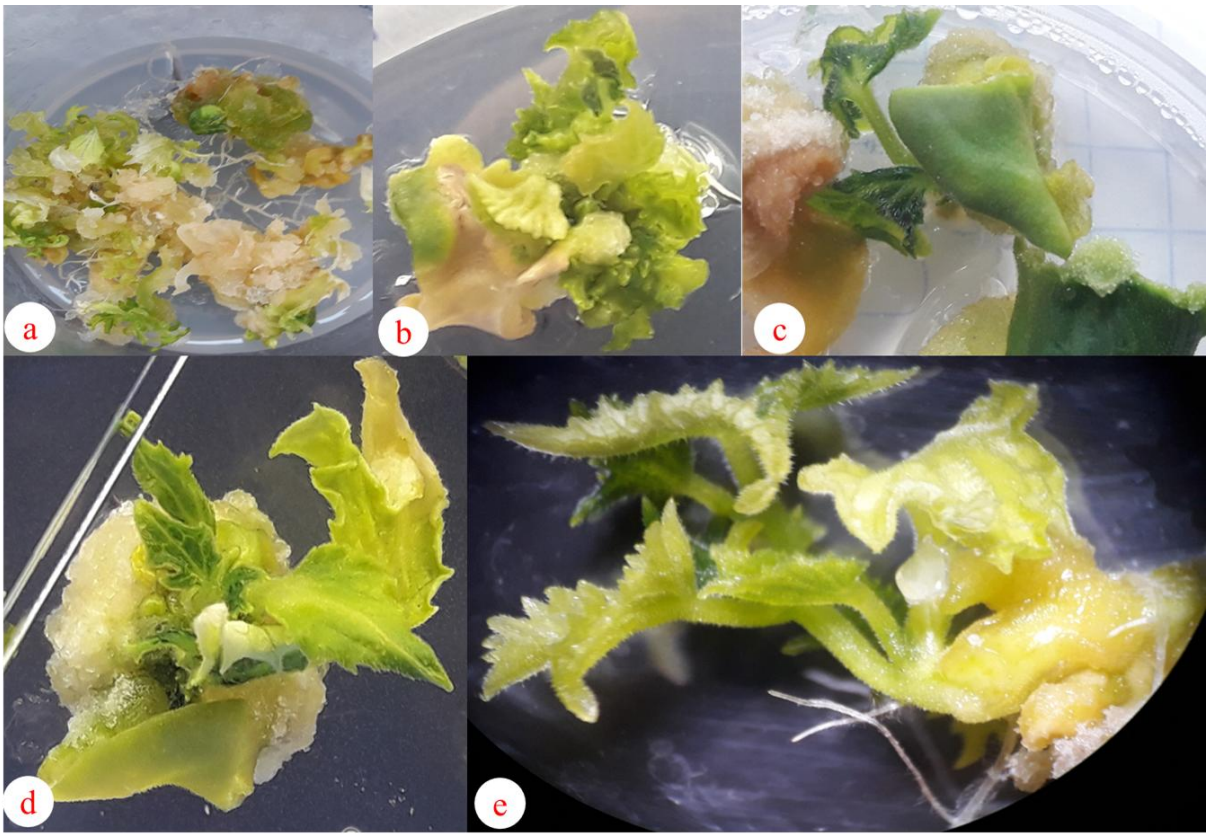

Figure 1. Callus induction and regeneration of adventitious shoots from cotyledon derived callus of *Cucumis sativus* in MS medium. a Induction of embryogenesis, organogenesis and leaf like structure from cotyledon derived calli, b initiation of adventitious shoots from greenish compact nodular callus, c -e direct regeneration from cotyledon

When Isfahan's hypocotyl explants were cultured in M5 medium containing 2 mg/l BAP and 0.1 mg/l NAA, 30% adventitious shoots were induced, whereas no regeneration occurred in other combinations of BAP and NAA. It can be concluded that while there was no significant difference in the effectiveness of different combinations of BAP and NAA in inducing hypocotyl and cotyledon callus, they led to more regeneration of cotyledon explants than hypocotyl. Therefore, it can be said that the cotyledons of the Esfahan genotype responded better to the tested compounds of BAP and NAA than the hypocotyl.

The hypocotyl cultures that responded the most, with a maximum frequency of multiple shoot bud induction (55%) for Beta alpha, were observed when 1 mg/L BAP was combined with 0.2 mg/L NAA (M3). The second highest response was observed in M1 (1 mg/L BAP and 0.05 mg/L NAA), followed by M4 (1 mg/L BAP and 0.5 mg/L NAA), M6 (2 mg/L BAP and 0.5 mg/L NAA), and M7 (3 mg/L BAP and 0.1 mg/L NAA), all with 30% cultures responding and producing shoots/explants. There were no observed responses to regeneration when any other BAP and NAA concentrations were tested for Beta alpha hybrid. The number of shoots was significantly reduced when the concentration of BAP was increased. Moreover, there was no response in M9 and M10 when 4 mg/l of BAP was used. On the other hand, increasing the concentration of NAA from 0.1 in M5 to 0.5 in M6 led to an increase in the regeneration induction (Table 1). During the testing of the beta alpha cultivar, it was observed that the hypocotyl had a higher regeneration rate when exposed to the tested combination of plant growth regulators compared to the cotyledon. Both direct and indirect regeneration were noted in the tested media using hypocotyl explant and calli derived from hypocotyl for both genotypes (Figure 2).

Table 1. Plant growth regulator combinations tested for cucumber cotyledon and hypocotyls culture, callus induction response and regenerated plants obtained in MS-based media for Esfahani and Beta Alpha genotypes.

			Isfahan				Beta Alpha			
			cotyledons		hypocotyl		cotyledons		hypocotyl	
Media	BAP	NAA	callus	regeneration	callus	regeneration	callus	regeneration	callus	regeneration
M1	1	0.05	100	30	100	0	100	0	100	30
M2	1	0.1	100	0	100	0	100	0	100	0
M3	1	0.2	100	0	100	0	100	60	100	55
M4	1	0.5	100	0	100	0	100	60	100	30
M5	2	0.1	100	10	100	30	100	0	100	0
M6	2	0.5	100	30	100	0	100	0	100	30
M7	3	0.1	100	10	100	0	100	0	100	30
M8	3	0.5	100	65	100	0	100	0	100	0
M9	4	0.1	100	60	100	0	100	0	100	0
M10	4	0.5	100	30	100	0	100	0	100	0

In a study conducted by Sultana *et al*. (2021), the regeneration of cotyledon, hypocotyl, and leaf disc of cucumber was tested. The study found that only cotyledon explants induced

adventitious shoots. The treatment combination of BAP at 2.0 mg/l, NAA at 0.2 mg/l, and 1.0 mg/l resulted in a high frequency of shoot regeneration. However, in our study, we observed that both cotyledons and hypocotyls of the Isfahan and Beta alpha genotypes induced a high rate of regenerated plants. According to Ugandhar *et al*. (2011), the highest number of shoot buds were produced when a higher concentration of BAP (3mg/l) was combined with a lower concentration of auxin (0.5mg/l). This finding supports our results. Similarly, Selvaraj *et al*. (2006) reported that a high frequency of plant regeneration was observed in cucumber when hypocotyl -derived callus was cultured on MS medium containing a combination of 2, 4-D and BA or a combination of BA, zeatin, and coconut water.

Present study, like the one reported by Kim *et al*. (1988), found that shoot regeneration from callus culture of cucumber cotyledons was dependent on genotype. There were notable differences observed between the Beta alpha hybrid and Iranian landrace Isfahan regarding the regenerated plants and types of explants. These differences highlight the significance of the genotypes background. According to the present study, different explants may display varying responses. The results indicate that cotyledons in the Isfahan genotype and hypocotyls in the beta-alpha variety exhibited better regeneration when tested with the mentioned plant growth regulators. It appears that each genotype requires specific callogenesis and regenerated media to achieve desirable results.

It is therefore evident that having a protocol to accelerate the proliferation of cucumber using tissue culture technology would be important. The findings indicate that it is possible to regrow and propagate cucumber using cotyledon and hypocotyl explants, which produce plantlets that have the same growth characteristics as the original plant. However, further cytological studies are required to determine the reasons why hypocotyl and cotyledon explants have the potential to regenerate cucumbers. The results of this study on the regeneration protocol are also expected to contribute to genetic transformation work in cucumber.

CONCLUSION

According to the findings of the present study, the compounds used as plant growth regulators induced callus induction from hypocotyl and cotyledon explants of cucumber in both genotypes in all culture media. However, the regeneration varied in different explants and genotypes, which may be due to the difference in internal hormone balance in different organs of the plant, depending on the genotype. In other words, each genotype has a certain concentration of internal hormones, and the response of its tissue to plant growth regulators depends on the internal hormone concentration and also the type of explants. Therefore, optimizing a specific culture media is necessary for each explant and genotype. It appears that each genotype requires specific callogenesis and regenerated media to achieve desirable results. Overall, the results suggest that cotyledons in the Isfahan genotype and hypocotyls in the Beta-alpha variety exhibited better regeneration when tested with these plant growth regulators. The study's results can pave the way for callus production and indirect reproduction of cucumber cultivars. This will be helpful in micropropagation goals, cell suspension, and transgenic studies. The research can also be useful in producing recombinant oral vaccines in this plant.

Figure 2. Callus induction and regeneration of adventitious shoots from hypocotyls derived callus of *Cucumis sativus* in MS medium. a hypocotyl with an emerging callus and callus mass after 12 days of inoculation, b-d initiation of adventitious shoots from greenish compact nodular callus cotyledon, e direct regeneration from hypocotyls and indirect regeneration from derived callus, f *in vitro* regenerated plant and g regenerated plant with male flower.

REFERENCES

Asadi A. Segui-Simarro J. M. 2021. Production of doubled haploid plants in cucumber (*Cucumis sativus* L.) through anther culture. Doubled Haploid Technology: Volume 3: Emerging Tools, Cucurbits, Trees, Other Species**,** 71-85.

Asadi A., Zebarjadi A., Abdollahi M. R. Segui-Simarro J. M. 2018. Assessment of different anther culture approaches to produce doubled haploids in cucumber (*Cucumis sativus* L.). Euphytica, 214**,** 1-17.

Gambley R. L. Dodd W. A. 1990. An in vitro technique for the production de novo of multiple shoots in cotyledon explants of cucumber (*Cucumis sativus* L.). Plant cell, tissue and organ culture, 20**,** 177-183.

Gemes-Juhasz A., Balogh, P., Ferenczy A. Kristof Z. 2002. Effect of optimal stage of female gametophyte and heat treatment on in vitro gynogenesis induction in cucumber (*Cucumis sativus* L.). Plant Cell Reports, 21**,** 105-111.

Kim J.-W., Han S.-K., Kwon S.-Y., Lee H.-S., Lim Y. P., Liu J. R. Kwak S.-S. 2000. High frequency shoot induction and plant regeneration from cotyledonary hypocotyl explants of cucumber (*Cucumis sativus L.*) seedlings. Journal of plant physiology, 157**,** 136-139.

Kim S.-G., Chang J.-R., Cha H. C. Lee K.-W. 1988. Callus growth and plant regeneration in diverse cultivars of cucumber (*Cucumis sativus* L.). Plant Cell, Tissue and Organ Culture, 12**,** 67-74.

Mashayekhi K., Sharifani M., Shahsavand M. Kalati H. 2012. Induction of somatic embryogenesis in absence of exogenous auxin in cucumber (*Cucumis sativus* L.). International Journal of Plant Production, 2**,** 163-166.

Mohiuddin A., Chowdhury M., Abdullah Z. C. NAPIS S. 1997. Influence of silver nitrate (ethylene inhibitor) on cucumber in vitro shoot regeneration. Plant cell, tissue and organ culture, 51**,** 75-78.

Selvaraj N., Vasudevan A., Manickavasagam M. Ganapathi A. 2006. In vitro organogenesis and plant formation in cucumber. Biologia plantarum, 50**,** 123-126.

Selvaraj N., Vasudevan A., Manickavasagam M., Kasthurirengan S. Ganapathi A. 2007. High frequency shoot regeneration from cotyledon explants of cucumber via organogenesis. Scientia Horticulturae, 112**,** 2-8.

Song J., Cao K., Hao Y., Song S., Su, W. Liu H. 2019. Hypocotyl elongation is regulated by supplemental blue and red light in cucumber seedling. Gene, 707**,** 117-125.

Sultana H., Nahar L., Hossain M. M., Ghosh T. K. Biswas M. S. 2021. Response of Different Explants for Callus Induction in Cucumber. European Journal of Biology and Biotechnology, 2**,** 71-75.

Ugandhar T., Venkateshwarrlu M., Begum G., Srilatha T. Jagamohanreddy K. 2011. In Vitro plant regeneration of Cucumber (Cucumis sativum (L.) from cotyledon and hypocotyl explants. Science Research Reporter, 1**,** 164-169.

Vasudevan A., Selvaraj N., Ganapathi A., Kasthurirengan S., Ramsesh Anbazhagan V. Manickavasagam M. 2004. Glutamine: a suitable nitrogen source for enhanced shoot multiplication in Cucumis sativus L. Biologia Plantarum, 48**,** 125-128.

Venkatachalam P., Jinu U., Sangeetha P., Geetha N. Sahi S. V. 2018. High frequency plant regeneration from cotyledonary node explants of Cucumis sativus L. cultivar 'Green Long'via adventitious shoot organogenesis and assessment of genetic fidelity by RAPD-PCR technology. 3 Biotech, 8**,** 1-12.

Venkatachalam P., Sangetha P., Geetha N. Sahi S. V. 2015. Phytofabrication of bioactive molecules encapsulated metallic silver nanoparticles from Cucumis sativus L. and its enhanced wound healing potential in rat model. Journal of Nanomaterials, 16**,** 241-241.