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# Callus induction and efficient plant regeneration in Cucumber (*Cucumis sativus* L.)

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## ABSTRACT

A reliable and reproducible protocol is established to get healthy and wellformed callus from juvenile explants of cucumber. The sterilized seeds of cucumber cultivar were cultured on MS basal medium (Murashige and Skoog, 1962). The seeds germinated after 7 days of culture with 24 hours dark photoperiod. Explants from germinated seedlings were cultured on MS medium supplemented with individual treatments of different auxins (2,4-dichloro-phenoxyacetic acid (2,4-D),  $\alpha$  naphthalene acetic acid (NAA)) or cytokinins (benzyl aminopurine (BAP)). Plant parts such as leaves, stems and cotyledons were used as source of explants. Callus were initiated from leaves, stems and cotyledons after 4 weeks of culture. The optimum medium for callus induction from leave, stem and cotyledon explants was MS medium supplemented with 0.5 mg/L BAP added with 1.0 mg/L NAA. The highest percentage of callus was obtained from stem explants ( $89.0 \pm 0.75$  %) followed by leave ( $79.05 \pm 3.28$ %) and cotyledon ( $74.43 \pm 1.30$  %) explants. Maximum callus induction in stems ( $73.05 \pm 2.1$ %) was obtained in 1.mg/l concentration of BAP. Incorporating 2,4-D in the callus induction media promoted slow callus growth and low quality callus compared to that produced on media containing NAA and BAP. Callus induced on media containing 2,4-D was friable and yellow in color. This protocol can promote the application of tissue culture technology to facilitate the genetic transformation of this species.

**Key Words:** Cotyledon, Naphatalene acetic acid, Benzylaminopurine, propagation and Explants

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## I. Introduction

Cucumber is a monoecious, annual, herbaceous and vining plant. Cultivated cucumbers are distributed throughout the world and are the fourth most important vegetable crop behind tomato, cabbage and onion (Tatlioglu, 1993). The genus *Cucumis* contains 52 species; of which cucumber and melon are the two most economically important food crops (Ghebretinsae et al., 2007). It is a popular vegetable crop of the family Cucurbitaceae, is rich in phosphorus, potassium and oxalic acid and is popularly used in salads. Its seeds are diuretic, tonic and refrigerant. It has strong antioxidant potential that exhibited chilling and soothing effect to irritated skin caused by sun or cutaneous

eruption (Budhiraja, 2014). The odorous principle of *Cucumis* L. is extractable with alcohol and is used in certain bouquet perfumes (Pandey, 2000).

Cucumber plants naturally thrive in both temperate and tropical environments, and generally require temperatures between 60-90°F/15-33°C. Commercial production of cucumbers is usually divided into two types. "Slicing cucumbers" are produced for fresh consumption. "Pickling cucumbers" are produced for eventual processing into pickles. The cucumber is thought to have been first domesticated in central Asia (Harlan, 1971). It is a popular crop in Bangladesh, cultivated primarily for its fruits for slicing and pickling, juice extraction and for the preparation of traditional local medicines. Conventional breeding programs and transfer of desirable traits, especially stress resistance from wild species have not been successful in *Cucumis* (Esquinas-Alcazer et al., 1983). The intention of modern biotechnology is the improvement of crops by means that are impossible by classical plant breeding (Gaba et al., 2004). In order to achieve the goal of modern biotechnology for the development of transgenic cucumber plant, successful and efficient *in vitro* regeneration system is required (Mohiuddin et al., 1997). The use of molecular biotechnology in cucurbits genetic improvement solved many problems associated with traditional breeding techniques (Flores et al., 2002).

*In vitro* regeneration of cucumber is possible using various culture techniques (Malepszy, 1988). A variety of explants have been used for plant regeneration from cucumber. Callus usually can be produced from any differentiated structure (e.g., Leaf, stem, root, cotyledon etc.) by placing explants on media containing relatively high level of auxin. Once produced, the callus can be grown either as large, multicellular masses on solid media or as small cell aggregates in rotated liquid media (El-Bahr et al., 2001). Kim et al. (1988) obtained the callus of ten cultivars of cucumber on Murashige and Skoog (MS) medium supplemented with 2, 4-D and BA. A variety of explants have been used for plant regeneration from cucumber via organogenesis viz., cotyledons (Chee, 1990), primary leaves (Seo et al., 2000), petioles (Punja et al., 1990) and hypocotyls (Selvaraj et al., 2006). Plant cell and organ culture is fast developing in the field of secondary metabolism regulation, with the development of molecular biology, deepen understanding of the biosynthetic pathways of natural products and newly developed treatment strategies (Gaosheng and Jingming, 2012). Several protocols for the genetic transformation of cucumber (Ahmad et al., 2005; Chee and Slightom, 1991; Sarmiento et al., 1992; Nishibayashi et al., 1996; Raharjo et al., 1996) have been established utilizing indirect organogenesis procedures.

Despite the availability of various publications on cucumber organogenesis and genetic transformation, a careful perusal reveals that the frequency of regeneration or transformation was comparatively low and it was dependent to a greater extent on the type of the explants, the cultivars, hormone combinations and physical conditions of culture. Wehner and Locy (1981) and Kim et al. (1988) already indicated that the success of cucumber regeneration was largely genotype dependent. Therefore, the aforesaid facts constantly call for improved regeneration protocols for cucumber genotypes for their genetic improvement. Accordingly, the present work was undertaken to establish an efficient and reproducible protocol for callus induction from stem, leaf and cotyledon explants of commercially important slicing cucumber Hatiyakira.

## II. Materials and Methods

**Seed sources and sterilization procedures:** The seeds of cucumber cultivar Hatiyakira, were obtained from Horticultural Centre, BSMRAU. Seeds were rinsed under running tap water for thirty minutes and then rinsed twice with distilled water. Subsequently, the seed coat was removed. After that, the seeds were surface sterilized with 70% ethanol for 30 seconds respectively followed by rinsing with sterilized distilled water for five times followed by 0.1% HgCl<sub>2</sub> for 5 minutes. Finally, the seeds were rinsed three times with sterile distilled water. Rinsing of seeds with 70 % ethanol was done in the laminar flow (under aseptic conditions).

**Culture procedures:** The sterilized decoated seeds were then placed on MS basal medium (Murashige and Skoog, 1962) solidified with agar for germination. Culture were incubated in dark photoperiod at 26° C. Seeds were germinated after 7 days of culture. Prior to germinating, cultures were transferred

to cool-white-fluorescent light room and incubated at  $25 \pm 1^\circ \text{C}$  with 16 hours light and 8 hours dark photoperiod.

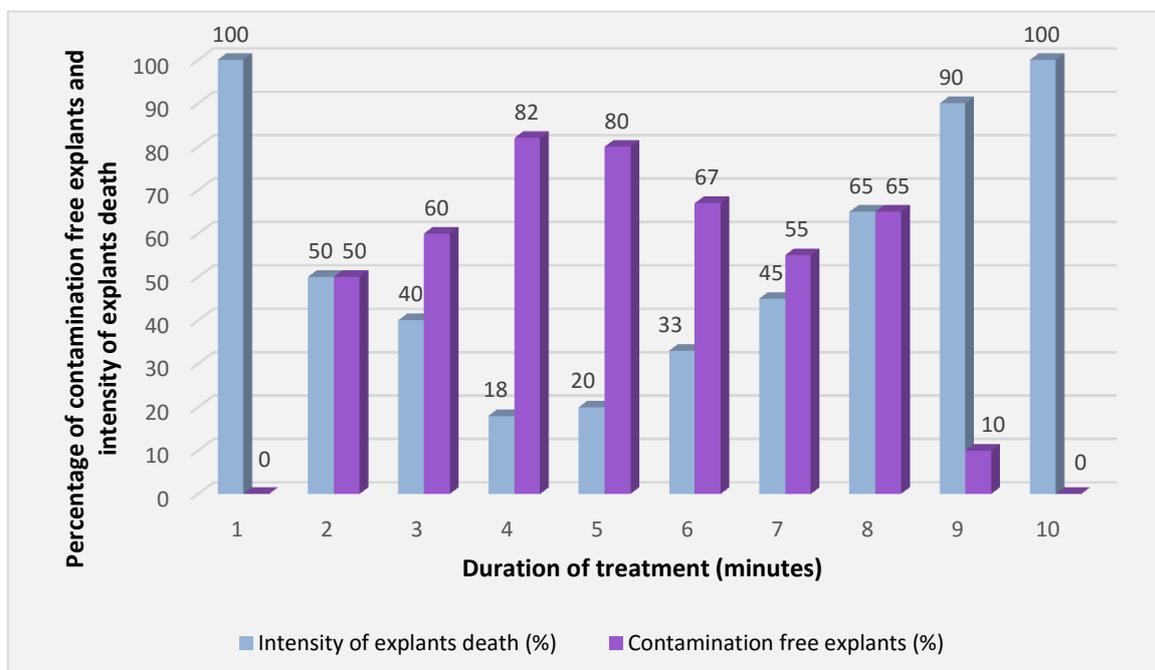
**Explant isolation and media composition:** Leaf, stem, and cotyledon explants excised from *in vitro* plantlets containing were then placed on MS medium containing 30 g/L sucrose supplemented with various concentrations of 2, 4-D (2, 3, 5 mg/L), BAP separately (0.5, 1.0, 1.5 mg/L) and in a combinations of BAP and NAA (0.5 + 1.5, 0.5 + 1.0, 1.0 + 0.5 mg/L, respectively) (Tables 01). Media were solidified with 8 gram agar and sterilized in an autoclave for 20 minutes at  $121^\circ \text{C}$  at 15 psi pressure.

**Culture conditions:** The pH of media was adjusted to 5.8 prior to autoclaving. All cultures at  $25 \pm 1^\circ \text{C}$  under white fluorescent light of  $40\text{-}60 \mu\text{mol m}^{-2}\text{s}^{-1}$  intensity for 16 hours light/8 hours dark photoperiod were incubated.

**Experimental layout:** The experiments were laid out in completely randomized design (CRD) with at least three replications. All data obtained were statistically analyzed using Microsoft Excel. The data gathered from the experiments were analyzed according to mean percentages and analysis variance (ANOVA) at 5% level of significance. Total number of explants in each treatment was 60. The experiments was conducted twice.

### III. Results and Discussion

The employment of biotechnology in plant improvement is dependent on callus induction and subsequent plant regeneration (Murphy, 2003). The success in callus induction is affected predominantly by the type of explant material and the *in vitro* culture conditions (Ozgen et al., 1998). Callus regeneration is advantageous over direct regeneration for genetic transformation, since effective selection of cells having the transgene can be achieved (Radhakrishnan et al., 2007). Different species of Cucurbitaceae have difference in micro propagation. The difference might have arisen due to many other factors like- genotypes, medium composition, physical growth factors like- light, temperature, moisture etc.

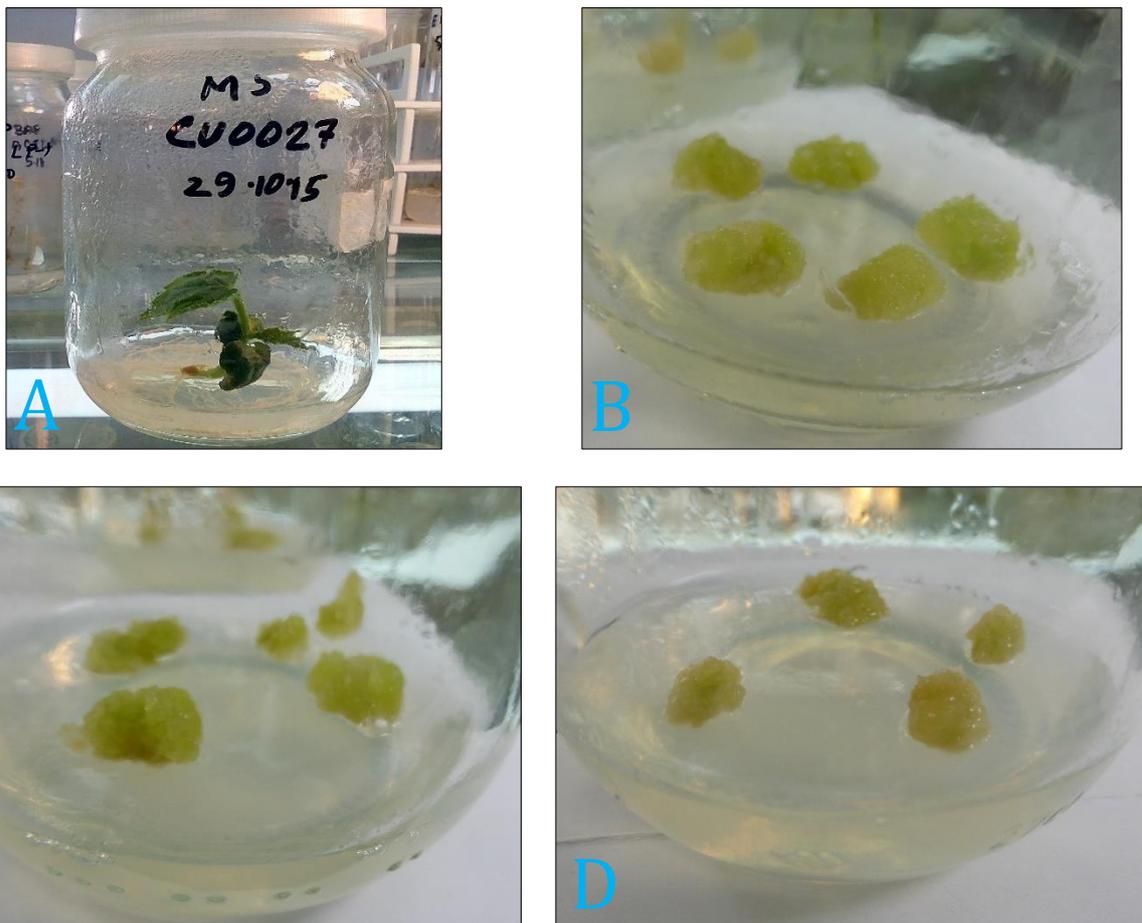


**Figure 01. Contamination free explants and intensity of explants death after treatment of  $\text{HgCl}_2$ .**

For the establishment of *in vitro* culture, surface sterilization of explants was mandatory for the medium used in these techniques are also suitable for microbial growth.  $\text{HgCl}_2$  was optimized for the surface sterilization because the chlorine gas released from  $\text{HgCl}_2$  was very penetrating that it destroyed the microorganisms present in most tissue of the explants. In this attempt of surface

sterilization with 0.1% (HgCl<sub>2</sub>) for treatment duration of 1 minute showed 80% of contamination. Maximum cultured explants showed fungal contaminations within 3 to 4 days for incubation. About 82-80% contamination free cultures were obtained when the explants treated for 4-5 minutes with 0.1% HgCl<sub>2</sub> (Figure 01). In this case, most of the explants were showed green and healthy growth and formation of auxiliary shoots. But 100% of the explants died when treated with HgCl<sub>2</sub> for 10 minutes.

Seeds of cucumber cultivar viz. Hatiyakira responded positively for germination *in vitro* when cultured on MS medium. Hatiyakira cultivar seed 28% germinated, on the 2nd day of culture and 100% germination percentage was obtained in 12-15 days of culture. In this study, regeneration of *Cucumis sativus* was successfully obtained when sterilized seeds were cultured on MS basal medium. Germination of aseptic seedlings was observed after 15 days of culture. Different explants from cucumber cultivars were explored for callus induction response on different media, however, no significant genotypic differences were observed.



**Figure 02 (A, B, C, D). A- 15 days old in vitro seedling of *Cucumis sativus*; (B-D)- Callus induction and proliferation response after 4 weeks of culture on MS medium containing NAA+ BAP, BAP and 2, 4-D respectively.**

Callusing ability of leaves, stems and cotyledons explants derived from 15-day-old in vitro seedlings (Figure 02). Callus of *Cucumis sativus* was induced on MS medium supplemented with individual treatments of different concentration of 2, 4-D, BAP and NAA + BAP (Table 01). Data were analyzed after four weeks of culture and the analysis showed that callus induction frequency, callus growth rate and nature of callus were affected by the type and concentration of the plant growth regulators. Callusing occurred at the cut ends of the leaves, stems and cotyledons after six days of culture initiation. According to Dixon and Gonzales (1994), inclusion of an auxin will be necessary for callus growth and somewhat higher auxin concentration may be required for callus initiation.

The optimum callus induction frequency was observed on MS medium fortified with NAA + BAP (1.0mg/l+ 0.5 mg/l) (Table 01). It gave a considerable level of callus induction, resulted in  $89.0 \pm 0.75$  % of stems,  $79.5 \pm 3.28$  % of leaves,  $74.43 \pm 1.30$ % of cotyledons were culture showing callusing. NAA at 1.0 mg/l showed the best callus. Several types of calli were distinguishable based on the physical appearance. Callus induced on MS medium containing NAA (0.1-1.0 mg/l) was compact and green in color (Figure 02). Among three concentration of BAP, maximum callus induction in stems ( $73.05 \pm 2.1$ %) was obtained in 1.mg/l concentration. The callus induced in this media was also compact and green in color (Figure 02). 2, 4 -D in 2.0 mg/l concentration showed the best performance in case of callus induction among three concentrations and the callus was compact and yellow in color (Figure 02). Further increase in the PGRs showed decline in callus induction. Overall among different explants explored for callus induction on different media, the highest callus induction was observed in stem explant followed by leaf and cotyledon explant on MS medium containing NAA+BAP (1.0 + 0.5 mg/L) (Figure 03).

**Table 01. Callus induction percentage in different explants on MS media supplemented with different plant growth regulators (PGRs)**

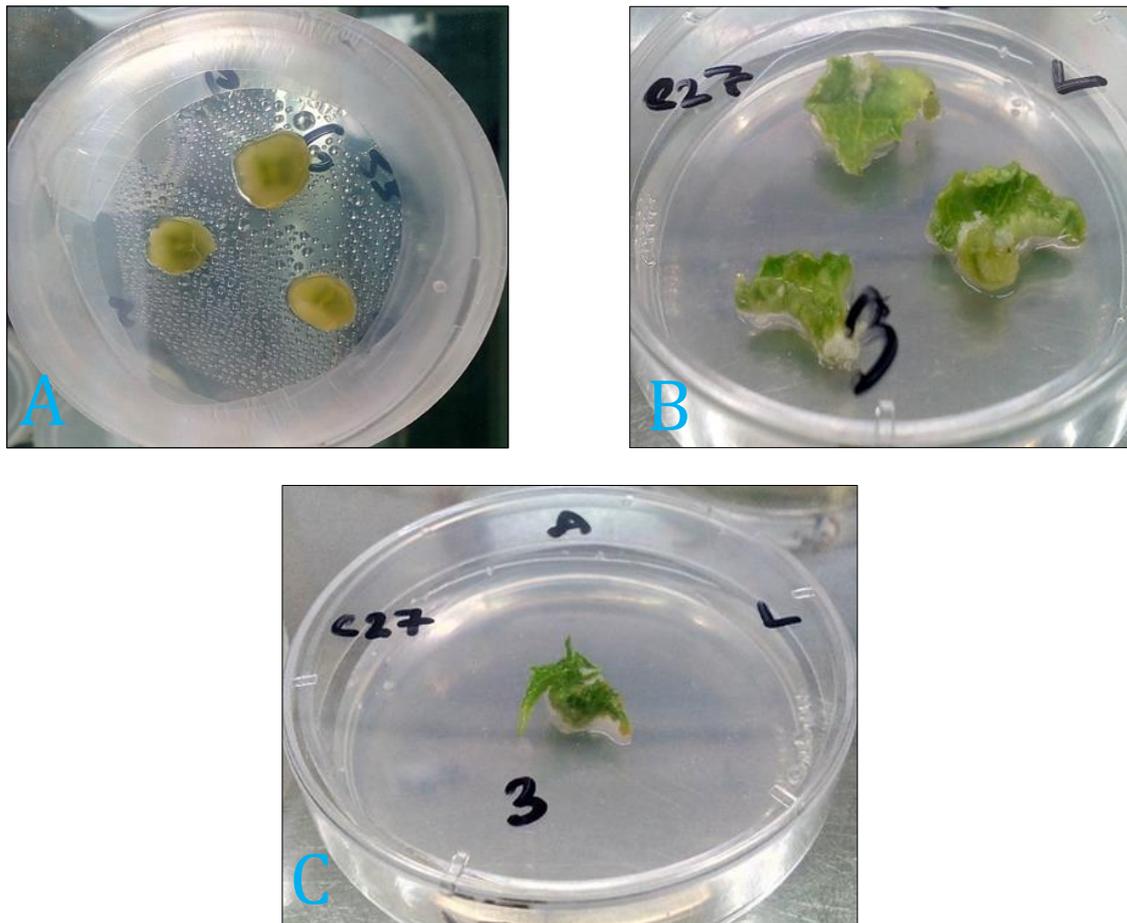
PGRs Treatments		Leaves	Stems	Cotyledons	Nature of callus
Control	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	
2, 4 -D	2.0 mg/l	51.00 ± 1.32	65.15 ± 1.12	50.33 ± 1.16	YC
	3.0 mg/l	46.00 ± 1.59	63.90 ± 1.39	43.83 ± 1.66	YF
	5.0 mg/l	45.03 ± 1.63	62.61 ± 1.33	42.50 ± 0.80	YF
BAP	1.0 mg/l	55.00 ± 1.44	62.01 ± 1.10	54.75 ± 1.25	GC
	1.5 mg/l	61.00 ± 1.54	70.05 ± 2.10	59.33 ± 1.55	GC
	0.5 mg/l	51.75 ± 1.87	59.35 ± 1.44	51.05 ± 0.80	YC
NAA+BAP	0.5 +1.0 mg/l	74.00 ± 1.38	83.76 ± 1.09	71.00 ± 1.13	YC
	0.5 + 1.5 mg/l	76.00 ± 1.44	84.75 ± 1.12	72.00 ± .80	GC
	1.0 + 0.5 mg/l	79.05 ± 3.28	89.00 ± 0.75	74.00 ± 1.30	GC

B: brown; G: green; Y: yellow; C: compact; F: friable.

Significantly greater callus were induced on various levels of 2, 4-D, BAP and BAP in combination with NAA. Our results are in accordance with the findings of Kim et al. (1988) and Ali et al. (1991) who induced callus from cotyledon of Burpless hybrid cucumber and each cotyledon was divided in 2, 4, 6 and 24 pieces. The findings of Lou & Kako (1994) and Ladyzynski et al. (2001) who developed an efficient method for obtaining callus from cotyledon, hypocotyls and first leaves in cucumber are in agreement as significantly higher calli induced on higher levels of 2,4-D. However, our results are in conformity with the findings of Alsop et al. (1978) and Punja et al. (1990) who observed callus induction from cotyledon explant. Our findings are further supported by the observations of Gambley & Dodd (1990) who obtained callus from cv. Crystal Salad hybrid cucumber. However, our results are contrary to the findings of Mazlan et al. (2014) who obtained highest percentage of callus from stem explants on different concentration of NAA and BAP combinations.

Development of an efficient callus culture and plant regeneration protocol for cucumber is the first step towards the application of genetic engineering to facilitate cucumber breeding strategies. Genetic transformation of this species is desirable for the development of new varieties that are resistant to biotic and abiotic stresses. Our result of developing embryogenic callus compared to other explants

make this protocol favorable for efficient multiplication of cucumber plants. Such protocols could be helpful for getting regeneration in transformed tissues on selective medium for developing transgenic plants in this economically important member of Cucurbitaceae family as well.



**Figure 03 (A, B, C). Formation of callus of stems, cotyledons and leaves respectively in MS medium supplemented with 0.5 mg/L BAP added with 1.0 mg/L NAA.**

#### IV. Conclusion

Micro propagation and callus induction of *Cucumis sativus* L. was successfully obtained. In recent years, many investigations have been performed in establishing reliable regeneration protocol for important vegetable crops primarily because of both primary and essential steps for facilitating gene introduction and crop improvement. The present experiment showed that an effective protocol for micropropagation of *Cucumis sativus* depends on various key factors like choice of explants, surface sterilization, growth regulators and their combination at different concentrations. It is concluded that the manipulation of culture conditions using various combinations and concentrations of growth hormones and other adjuvants can provide a reproducible protocol and reduce the high costs of hybrid seed production. Stem explants has been identified as the more regenerative explants for induction of callus. Formation of callus of stems, cotyledons and leaves respectively in MS medium supplemented with 0.5 mg/L BAP added with 1.0 mg/L NAA was showed the best result. Studies of *Cucumis sativus* L. clonal propagation and callus induction could also be efficiently adapted for other crops in future research.

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