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Research Paper

Micropropagation and field establishment of Japanese cucumber *(Cucumis sativus* L.) genotype

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Abstract

Cucumber (*Cucumis sativus* L.) is a commonly consumed vegetable that is growing in popularity in Bangladesh. Therefore, an attempt was taken for *in vitro* multiplication and field establishment of Japanese cucumber (*Cucumis sativus* L.) from shoot tip and nodal explants of *in vitro* germinated seedlings. Various phytohormons were used individually and in combination on Murashige and Skoog (MS) semi solid medium to examine the better response for multiplication. The best shoot multiplication was observed in MS media supplemented with 4.0 mg/l BA after 30 days of culture. In this media, the highest shoot number (15.33) and shoot length (10.5 cm) were noted. The results of different hormonal treatments used in combination in this experiment were moderate on shoot multiplication. Comparatively cytokinins were better than auxins to proliferate multiple shoots. Rooting response was superior in MS media without growth regulators than those of employed on media having different hormonal treatments. The plantlets with well-developed roots were successfully transplanted to the field after acclimatization process. About 80% of the plantlets were survived and successfully produced flowers and fruits.

Keywords: Phytohormones, *In vitro*, Multiplication, Regeneration, Acclimatization.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is a widely grown herb which belongs to the cucurbitaceae family, and fourth most cultivated vegetable in tropic and subtropic region (Innark *et al*. 2013). It is mainly a salad crop which has several medicinal properties too (Ugandhar *et al*. 2011). Conventional plant breeding method is used generally to obtain a high yielding new plant variety, which is resistance to some disease and pests.

Cucumber (*C. sativus* L.) is conventionally propagated by seeds. Low seed germination, expensive seeds and disease susceptibility are the major problems faced by the commercial growers. The seedlings are very susceptible to fungal and bacterial rots. *In vitro* propagation of plantlets could hence be an alternative method for overcoming this constrains. Dong and Jia (1991) successfully produced *in vitro* plantlets of cucumber using callus derived from cotyledon. Ahad *et al*. (1994) reported a complete plant regeneration system of cucumber from the mature and immature axis. Sultana *et al*. (2004) produced *in vitro* plantlets of cucumber from the callus culture of leaf segments. All the *in vitro* raised plantlets produced normal fruits similar (homogenous and true-to-type) to the seed derived plants.

Cucumber (*C. sativus* L) is a crop of economic importance. It is common, not just in our country, but in the whole world. To make better *C. sativus* L., large amounts of hybrid seeds have been used each year. F1 hybrid *C. sativus* L. seeds are very costly. For better production of *C. sativus* L. rich farmers of developed countries use micropropagation of F_1 plants derived from hybrid seeds. Keeping this idea in mind, the present research work was undertaken with aiming to introduce Japanese cucumber in our country through *in vitro* techniques.

MATERIALS AND METHODS

Plant Materials

The seed of cucumber (*C. sativus* L.) was the source of experimental materials which was imported from Japan. The shoot tips and nodal segments from *in vitro* germinated seedlings were used for further experiment.

Methods

The F_1 seeds of cucumber were washed with tap water for a few minutes to reduce the dust and then transferred in another conical flask containing distilled water adding with 2-3 drops of Tween-80 and few drops of savlon then kept them 3-5 minutes, and were washed 1-2 minutes with distilled water. Inside the laminar airflow cabinet surface disinfections of seeds was done with 0.1 % $HgC1_2$ solution by gently shaking for 3-8 minutes. After exposure to this sterilant, the seeds were washed in several times with double distilled water to remove all traces of $HgCl_2$. Sterilized seeds were placed in test tubes containing MS0 (Murashige and Skoog 1962) medium, and were kept in dark for germination. After germination, the culture vessels were incubated in a growth chamber providing special culture environment. The culture was maintained at 25± 1 °C with light intensity varied from 2000-3000 lux. The photoperiod was maintained generally 16-hour light and 8-hour dark.

The culture tubes were checked daily to note the morphogenic response of different experiment conducted in the present investigation. When the inoculated seeds were developed with 2-3 leaves then they were sub cultured on MS semi-solid medium supplemented with different growth regulators such as BA, KIN, NAA and IAA for shoot multiplication. After 30-35 days of culture, inoculated shoots and nodal segments produced multiple shoots. Regenerated microshoots were excised and cultured on shoot elongation and root induction medium. For proper rooting, at first the multiple shoots were taken out from the test tubes aseptically on sterile tiles with a sterile surgical blade inside of laminar air flow and cultured into different culture media in order to develop proper shoot length and root.

Data on number of explants inoculated, percentage of explants responded, days to shoot initiation number of micro-shoots per explants, average length of micro-shoot and root, number of roots per micro-shoot were recorded.

RESULTS AND DISCUSSION

Now a days, research aims are focused to obtain the increased production, higher nutrition value, greater plant resistance to adverse weather, pathogenic agents as well as pests. Crop improvement is generally done through introduction, selection, hybridization as well as *in vitro* method. Traditional plant breeding methods have some limitations to achieve success in several agronomic traits. Most of them are very lengthy process. Recent discoveries in applied genetic and molecular biology develop some methods for crop improvement. Tissue culture technique is thus recognized as noble technique to generate genetic variability and has been proposed an excellent supplementary technique for plant, which accelerate breeding programs, through the use of new expended genetic variability.

Table 1. Standardization of (0.1%) $HgCl_2$ treatment period for surface sterilization of seed**.** In all cases, 20 seeds were placed for germination

Treatment period of $HgCl_2$ (in minute)	No. of seeds sterilize	Contamination rate after days						% of contamination free seed
		2 days	3 days	4 days	5 days	6 days	7 days	
2	20	-	4	6	8	16	20	0
4	20	-	3	5	10	12	18	10%
6	20	-	-	2	6	8	10	50%
8	20	-	-	-	1	2	4	80%
10	20	-	-	-	-	-	-	100*
12	20	-	-	-	-	-	-	100**
14	20	-	-	-	-	-	-	100***

- = No Contamination, * = Tissue killing 1-25%,

** = Tissue killing 26-50%, *** = Tissue killing 51-100%

Standardization of $HgCl_2$ (0.1%) treatment period for surface sterilization of seeds

To establish *in vitro* culture, surface sterilization of seed or other plant parts (explants) is essential for the aseptic cultures. Many workers have used different types of sterilizing agents with different concentrations for surface sterilization of explant, such as Mercuric chloride ($HgCl_2$) 1% solution of hypo chloride, 70% alcohol, 1% silver nitrate solution (Cassells 2012). Mercuric chloride ($HgCl_2$) is one of the effective surface sterilants and as considered as a potent surface sterilizing agent but its residual inhibitory effect is greater than the

other sterilizing agent commonly used in plant tissue culture (Bhojawany and Razdan 1983; Das and Mitra 1990 and Torres 1998). In the present study, it was observed that that surface sterilization with 0.1% $HgCl_2$ solution for 2 min resulted 100% contamination of explants, whereas 80% contamination free culture with the highest proliferation ability were achieved when seeds were treated for 8 minutes.. On the other hand, seed treated with 0.1 % $HgCl_2$ for 10 minutes or more caused high tissue killing (**Table 1**). This result indicates that the duration of sterilization period is important factor for effective surface sterilization without damaging living tissue.

Induction of multiple shooting from *in vitro* grown shoot tip and nodal explats cultures

The nutrient media are also important for the growth and multiplication of plants. MS medium were supplemented with different concentration of auxin and cytokinin either alone or in combinations to see the adventitious shoot proliferation in cucumber. Proliferation strictly depends on exogenous hormone supplementation. In absence of exogenous hormone, explant failed to induce adventitious shoot proliferation. In this investigation, an attempt was taken for *in vitro* multiplication of Japanese cucumber (*C. sativus*) from shoot tip and nodal explants of *in vitro* germinated seedlings. Various phytohormons such as NAA, IAA as auxin, and BA, KIN as cytokinin in different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) were used to examine the better response for multiplication from shoot tip and nodal explants of *in vitro* grown plants. The best shoot multiplication was observed in MS media supplemented with 4.0 mg/l BA after 30 days of culture from nodal explants, where 80% culture induced shoot multiplication The results of different hormonal treatments used in this experiment were moderate on shoot multiplication. At 4.0 mg/l BA containing media, the highest shoot number (15.33) and shoot length (10.5 cm) were also noted (**Table 3, Fig. 2**). Comparative to shoot culture, the nodal explants of *C. sativus* L. exhibited better performance to proliferate multiple shoots. Similar result were reported on *Holarrhena antydysenteric*a (Datta and Datta 1984) and *Prosopis juliflo*ra (Nandwani and Ramawat 1991). However, Abu-Romman *et al*. (2015) found that KIN was the most effective for shoot multiplication of cucumber.

Among the tested growth regulators, BA was comparatively more suitable for shoot length than KIN, NAA, IAA. BA is known to be the most effective PGR for stimulating axillary shoot production in different plant. For cucumber, only BA, a cytokinin is needed for the induction of multiple shoot formation for the nodal segments. No multiple shoot was produced from the nodal segment when they were cultured on basic MS medium (**Table 2 and 3**), but some of the nodal segments produced roots instead shoots. The addition of only BA into the culture medium was also found to be effective for *in vit*ro shoot multiplication of *Alpinia purpurata* (Chang and Criley 1993). However, some other plant species required more than one type of cytokinin for shoot proliferation such as *Tectona grandis* (Goswami *et al*. 1999). Present study reveals that micropropagation of hybrid cucumber (*C. sativus* L.) is possible in MS medium supplemented with growth regulators.

Table 2. Impact of MS0 medium and different concentration of BA, KIN, NAA and IAA in MS medium on multiple shoot proliferation from shoot tip culture. For each treatment 10 explant were cultured.

PGR	Media composition (mg/l)	% of explants induced shoot regeneration	No of shoot/explant ($\overline{x}$ ±S.E) after 30 days	Mean length of the longest root after 30 days in cm. ($\overline{x}$ ±S.E)
	MS_0	20	1.0	3.83±0.60
BA	1.00	30	2.66±0.66	3.15±0.08
	2.00	40	4.66±0.20	5.48±0.26
	3.00	50	5.00±0.58	6.39±0.07
	4.00	60	7.33±0.33	8.15±0.07
	5.00	40	5.00±0.58	6.00±0.17
KIN	1.00	20	2.20±0.76	3.10±0.42
	2.00	30	3.33±0.33	4.10±0.21
	3.00	40	5.00±0.58	4.04±0.10
	4.00	50	6.66±0.33	5.09±0.29
	5.00	30	4.33±0.33	3.21±0.17
NAA	1.00	40	2.00±0.58	3.97±0.39
	2.00	50	3.00±0.12	4.34±0.53
	3.00	60	4.33±0.88	5.83±0.27
	4.00	50	3.33±0.33	3.07±0.12
	5.00	30	2.33 ±0.33	2.90±0.10
IAA	1.00	30	2.00±0.58	2.18±0.35
	2.00	40	3.00±0.58	3.23±0.18
	3.00	50	3.44±0.88	4.57±0.32
	4.00	40	2.88±0.58	3.88±0.21
	5.00	20	1.00±0.58	2.34±0.14

Table 3. Impact of MS0 medium and different concentration of BA, KIN, NAA, and IAA in MS medium on multiple shoot proliferation from cultured nodal segments. For each treatment, 10 explants were cultured.

PGR	Media composition (mg/l)	% of explants induced shoot regeneration	No of shoot/explant ($\overline{x}$ ±S.E) after 30 days	Mean length of the longest root after 30 days in cm. ($\overline{x}$ ±S.E)
	MS0	20	1.0	5.83±0.60
BA	1.00	30	5.33±0.88	5.17±0.09
	2.00	30	7.63±0.88	6.39±0.07
	3.00	50	10.00±0.58	6.00±0.17
	4.00	80	15.33±0.33	10.5±0.29
	5.00	70	9.33±1.76	7.10±0.14
KIN	1.00	20	4.67±1.45	3.10±0.21
	2.00	40	6.00±0.58	4.04±0.10
	3.00	50	8.50±2.08	5.09±0.29
	4.00	60	10.33±2.40	6.21±0.17
	5.00	40	7.67±0.67	5.97±0.39
NAA	1.00	30	4.33±1.33	5.34±0.53
	2.00	50	5.67±0.33	6.17±0.09
	3.00	60	6.33±1.20	4.07±0.12
	4.00	50	5.00±1.15	6.18±0.10
	5.00	10	4.33±0.88	4.90±0.35
IAA	1.00	40	3.00±1.20	3.23±0.18
	2.00	50	4.67±0.88	4.57±0.32
	3.00	60	7.33±1.33	5.13±0.19
	4.00	30	3.67±1.20	4.34±0.14
	5.00	20	3.33±0.88	3.29±0.02

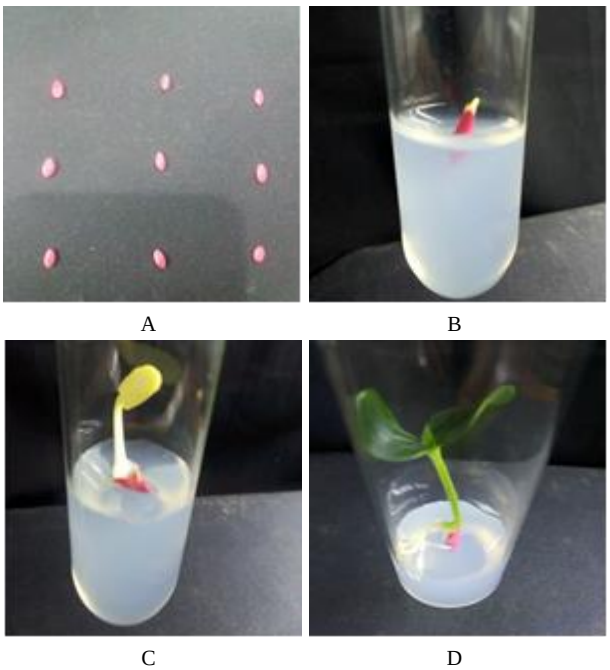

Fig. 1 Establishment of *in vitro* plants through germination of hybrid seeds of *C. sativus* L. A. Hybrid seeds of cucumber; B, C and D. Germination of seeds into MS medium.

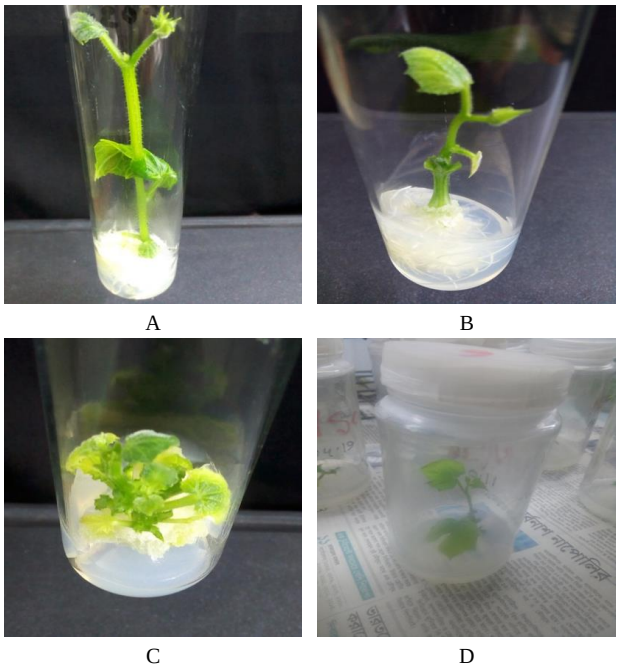

Fig. 2 Multiple shoot proliferation from *in vitro* derived shoot tip and nodal explants. A. Single shoot proliferation from tip explant in MS0 medium B. Single shoot proliferation from nodal explant in MS0 medium C and D. Shoots multiplication on MS medium supplemented with 4.0 mg/1BA after 30 days of culture.

Table 4. Impact of MS0 and different concentration of IBA in MS medium for root induction.

PGR	Media composition (mg/l)	Morphogenic response	
		Mean no of roots after 30 days ($\overline{x}$ ±S.E)	Mean length of the longest root after 30 days in cm ($\overline{x}$ ±S.E)
IBA	MS0	6.33±0.33	9.03±0.44
	0.5	3.33±0.67	4.29±0.03
	1.0	4.67±0.33	5.29±0.03
	1.5	3.33±0.33	4.60±0.06
	2.0	3.67±0.20	4.27±0.01
	2.5	3.33±0.67	4.24±0.01
	3.0	5.00±0.58	5.81±0.29

Root induction and field establishment

The micro cutting were induced to develop roots when they were subcultured into either MSO medium or supplemented with the different concentrations of auxins IBA. Subcultured microcuttings were induced to develop healthy root into MSO medium. Whereas, when the culture media contained auxin, micro cutting were induced to develop callus at base along with root (**Table 4, Fig. 3A**). Similar results were also reported in *Fragaria* × *ananassa* (Karim *et al*. 2015). Development of callus at base was eventually reduced field survibility of many of the plantlets. After two weeks of acclimatization in a container covered with punched

holes plastic sheets, the plantlets with well- developed roots were successfully transplanted to pots containing topsoil plus dark organic soil in the ratio of 2:1. Eighty percent of the acclimatized plantlets were survived after transplanting on to pots (Figure 3). Field performance of these plantlets showed some variation with the control plant. This result could vary according to the environment purpose (soil, pH, day length, temperature etc.) and with the cultivation techniques (irrigation, fertilizer, mulching, pruning, etc.). If this Japanese cucumber plant is cultured every year, it may be well- adapted in our country and enriched agriculture and economic sector, and in that case in vitro propagation may be important technique to introduce this crop in country.

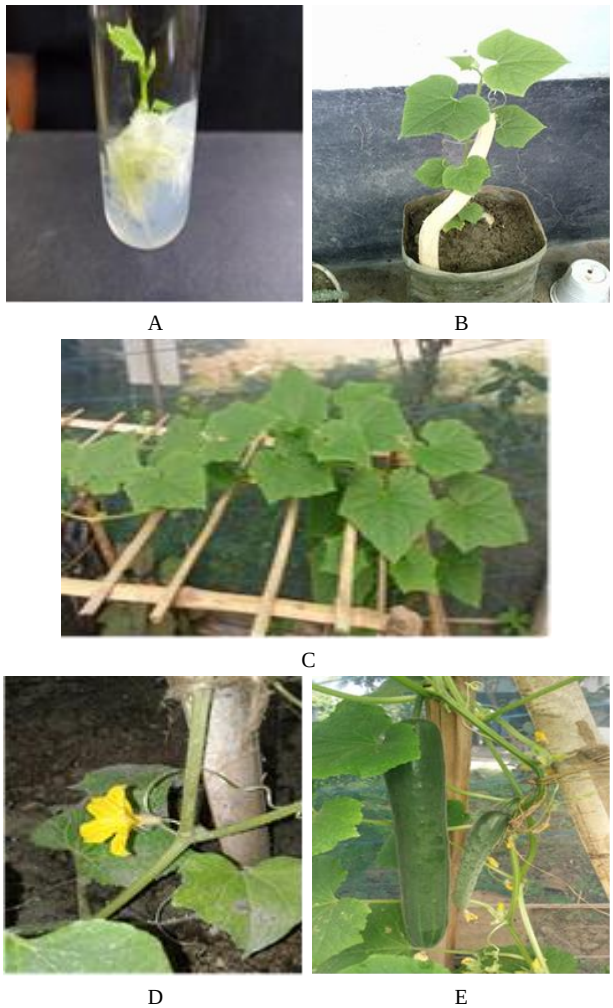

Fig. 3 Rooting, acclimatization and field establishment of Japanese cucumber (*C. sativus* L.) plantlets. A. Well-rooted plantlets in test-tube. B. Plantlets transplantation on pot under *ex vitro* condition. C. Transferred into field after 15 days. D. Mature plant after 40 days with flower. E. Mature plant with fruit after 82 days.

AUTHOR CONTRIBUTION

The experiment was designed and investigated by R. Karim and R. Khatun. R. Karim have written the main manuscript. R. Khatun, M. Rahman, M. Nasiruddin, M.H. Rahman and M. Hossain have helped to analyze the data and to edit the manuscript. All the authors read the final version of the manuscript, and there is no conflict of interests.

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