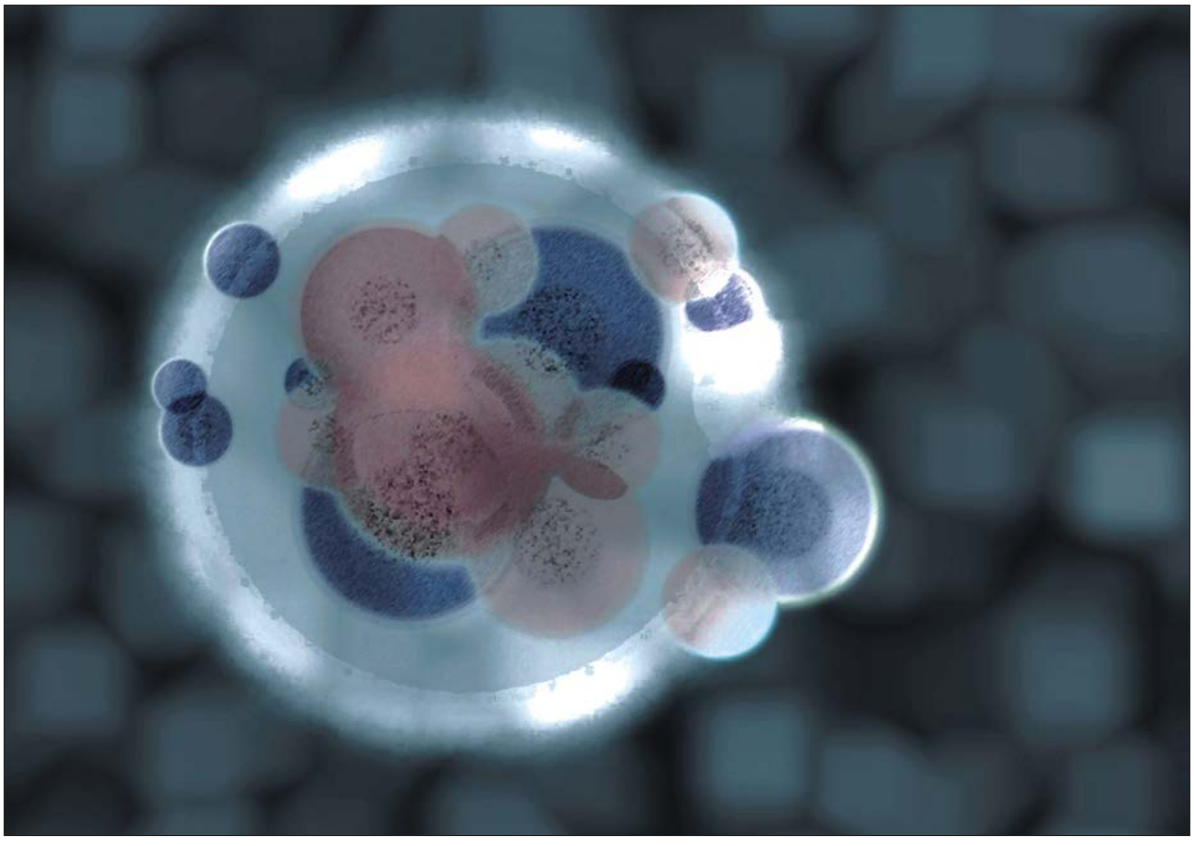

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# *In vitro* Clonal Propagation of *Cucumis sativus* L. By Shoot Tip Culture

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**Abstract:** The possibility of *in vitro* clonal propagation of *Cucumis sativus* L. cultivars Telegraph 314, Pepinex 69 and Rebella by the use of shoot tip explants was investigated as an interesting strategy for reducing the cost of hybrid seed production. Shoot tip explants (3 to 5 mm length) from 13-day-old *in vitro* grown seedlings were cultured on Murashige and Skoog (MS) medium supplemented with different BA and NAA concentrations (0.0, 0.3, 0.4 and 0.6 μM) and their combinations for 32 days. To investigate the effect of BA (6-Benziladenine) and NAA (• -Naphthaleneacetic acid) on shoot proliferation the medium supplemented with 0.0, 0.3, 0.4 and 0.6 μM of each Plant Growth Regulator (PGR) alone and in combination. The proliferation rate, shoot quality and other parameters studied indicate that the optimal treatment was 0.4 μM BA. The proliferated shoots succeeded to root in each treatment with different frequencies (45-100%) although the highest rooting frequency was obtained from the PGR-free medium. The plantlets were gradually acclimated *ex vitro* and successfully established under greenhouse conditions.

**Key words:** *Cucumis sativus* L., clonal propagation, shoot tip explants, shoot proliferation

## INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an important cultivated species of Cucurbitaceae. In the world, cucumber is grown on 2.485 million hectares with a total production of 42.639 million tons (Anonymous, 2005). It has significant value as food crops in tropics, subtropics and milder portions of the temperate zones of both hemispheres. Nutritionally, the cucumber has a relatively high mineral content and it has been reported as a functional food due to enhanced drug detoxification effect and antioxidant activity (Milner, 2000; Chu *et al*., 2002).

Many important crop plants are propagated vegetatively and grown as clones, however cucumber is usually propagated by seeds. A good micropropagation protocol for cucumber could be used for reducing the cost of hybrid seed production, which can account for more than 30% of the total seedling cost (Konstas and Kintzios, 2003). The regeneration of cucumber plants has been reported either directly from nodal (Konstas and Kintzios, 2003; Ahmad and Anis, 2005) and shoot tip (Vasudevan *et al*., 2004) explants or via callus that developed on anthers (Kumar *et al*., 2003), cotyledons (Zhu and Chen, 2005), hypocotyls (Selveraj *et al*., 2006), leaves (Burza and Malepszy, 1995a), petioles (Punja *et al*., 1990) and protoplasts (Burza and Malepszy, 1995b). However, a large scale somaclonal variation in tissue culture derived regenerants was found when plants were regenerated indirectly through somatic embryogenesis, cell suspensions or isolated protoplast culture (Debeaujon and Branchard, 1993; Plader *et al*., 1998; Ladyzynski *et al*., 2002; Filipecki *et al*., 2005). Indirect shoot regeneration is also time consuming. In contrast, direct shoot regeneration (organogenesis) is less time consuming with less abnormality observed in the regenerants (Mohiuddin *et al*., 1997). Therefore, regeneration via direct organogenesis (for example, from nodal and shoot tip explants) is frequently preferred for commercial, mass propagation purposes. With the aim of developing a reliable clonal propagation protocol, the present study was undertaken to investigate the effect of different auxin and cytokinin concentrations and combinations on *in vitro* morphogenesis of cucumber from shoot tip explants.

## MATERIALS AND METHODS

The seeds of cucumber cultivars Telegraph 314 (improved), Rebella (F1) and Pepinex 69 (F1) were surface-disinfected with 70% ethanol for 1 min and 4% sodium hypochlorite (10-14% w/v available chlorine) containing 5 drops of tween 80 for 5 min followed by three rinses with sterile distilled water. Disinfected seeds were aseptically germinated on half strength

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MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar in 9 cm Petri dishes. Shoot tip explants (3 to 5 mm length) from 13-day-old *in vitro* grown seedlings were excised and transferred onto a 175 mL culture vessels containing 30 mL shoot growth medium. The shoot growth medium was MS medium with 3% sucrose and 0.7% agar. To investigate the effect of 6-Benzyladenine (BA) and •-Naphthaleneacetic acid (NAA) on shoot proliferation the medium supplemented with 0.0, 0.3, 0.4 and 0.6 μM of each Plant Growth Regulator (PGR) alone and in combination. In the experiments each single PGR treatment consisted of 10 replications and all experiments were repeated twice.

The pH of all culture media was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 25±1°C with a 16 h photoperiod provided by cool white fluorescent tubes with light intensity of 2.9 Wm•$^{2}$.

The proliferation rate [total number of new shoots and nodes with expanded internodes (>5 mm) per explant] and shoot length was recorded after a single culture passage of 32 days. The treatments were also ranked (1 to 16; best to worst) according the shoot quality, taking into account the amount of unwanted callus production, the lengths of the internodes and the general appearance of the plantlets. In addition, percentage of root and callus formation was recorded. Extent of root and callus formation was scored on an ascending scale from 0 to 5.

Rooted plantlets were washed with tap water and transplanted into plastic trays containing a mixture of sphagnum peat and per lite (1:1) and irrigated with tap water regularly. The plantlets were covered with polyethylene bags to maintain high humidity and acclimated at 25±1°C with a 16 h photoperiod. After 10 days, established plantlets were transferred to greenhouse (25°C).

## RESULTS

The results show that, it was possible to obtain 100% shoot regeneration response with high proliferation rates in each cucumber cultivar by the use of shoot tip culture on either PGR containing media or even on the PGR-free (control; MS basal medium) ones (Table 1). However, the explants on PGR-free medium produced unbranched shoots with an increase in the number of nodes in all the cultivars. The geotropic response of these shoots with very long internodes was usually abnormal.

Addition of BA to the basal MS medium resulted in multiple shoots per explant in each cultivar and the proliferation rate increased due to increased BA concentrations (Table 1). Nevertheless, the highest number of vigorous shoots was obtained on 0.4 μM BA. Increasing the BA concentration from 0.4 to 0.6 μM decreased the shoot vigor from 100 to 92%. In addition, both 0.3 and 0.6 μM BA treatments were induced vitrification.

An increase in proliferation rate of cucumber was also observed with the 0.3 μM NAA treatment (Table 1). However, both 0.4 and 0.6 μM NAA treatments caused a significant reduction in proliferation. This effect was more obvious in cvs. Telegraph 314 and Rebella.

The proliferation rates obtained in combined treatments of both PGR varied due to the concentration of BA and NAA in the applied combination. In cvs. Telegraph 314 and Rebella, the inhibitory effects of higher NAA concentrations (0.4 and 0.6 μM) were also observed in combined treatments and the best proliferation rates were obtained when higher concentration of BA and lowest concentration of NAA (0.6 μM BA+0.3 μM NAA) combined together. In cv. Pepinex 69, combined PGR treatments enhanced proliferation compared to control, but the rate of proliferation was lower than that of the 0.4 μM BA treatment.

The PGR treatments had no effect on shoot length in cv. Telegraph 314 as shown in Table 1. However, in cvs. Pepinex 69 and Rebella, there were significant differences in the length of shoots obtained in the proliferated cultures at the end of the study. The tallest shoots were achieved with PGR free medium. In both cultivars, PGR induced shoot growth was also observed on media supplemented with 0.6 μM BA, 0.3 μM and 0.6 μM NAA when compared to control. Additionally, 0.6 μM NAA plus 0.3 μM BA treatments and 0.6 μM BA plus 0.3 μM NAA treatments resulted with the longest shoots as observed in PGR free medium in cv. Pepinex 69 and cv. Rebella, respectively.

With regard to shoot quality, in each cultivar, the addition of BA and NAA to the culture medium alone or their combinations at high concentrations resulted in the worst shoot quality, whilst the 0.4 μM BA treatment gave the best quality cluster (Table 1). The optimum shoot development was also obtained on the medium containing 0.3 μM NAA alone or in combination with 0.3 μM BA. One of the characteristics taken into account as ranking the PGR treatments according to shoot quality was the amount of unwanted callusing. The occurrence of basal callus on PGR-free medium was cultivar dependent that 50% of explants in cv. Telegraph 314 produced callus. Conversely, the addition of BA and NAA alone or in combination to the culture media caused the explants to enhance callusing in all cultivars and the extent of callusing did not vary depend upon PGR type or concentration. Therefore, the effects of PGR treatments

Table 1: The effects of plant growth regulators on shoot induction from shoot tip explants of cucumber cultivars in MS medium. Values represent mean±standard error of 10 replicates per treatment in 2 repeated experiments

| | Cultivars | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Telegraph 314 | | | Pepinex 69 | | | Rebella | | |
| PGR treatments (μM) | Proliferation rate | Shoot length (cm) | Ranking by shoot quality | Proliferation rate | Shoot length (cm) | Ranking by shoot quality | Proliferation rate | Shoot length (cm) | Ranking by shoot quality |
| PGR-free* | 3.6±0.4 | 3.8±0.7 | 1 | 4.4±0.3 | 4.8±0.9 | 1 | 4.3±0.3 | 3.4±0.7 | 1 |
| 0.3 BA | 4.8±0.5 | 2.1±0.3 | 9 | 4.5±0.9 | 1.8±0.3 | 4 | 4.8±0.4 | 2.0±0.2 | 6 |
| 0.4 BA | 5.1±0.7 | 2.6±0.6 | 2 | 8.3±0.7 | 2.7±0.4 | 2 | 4.9±0.5 | 2.6±0.4 | 2 |
| 0.6 BA | 6.3±0.8 | 2.7±0.5 | 12 | 7.1±0.5 | 3.8±0.4 | 10 | 5.7±0.4 | 3.9±0.4 | 10 |
| 0.3 NAA | 5.5±0.8 | 2.9±0.6 | 7 | 5.7±0.3 | 3.6±0.5 | 3 | 5.3±0.5 | 3.1±0.4 | 5 |
| 0.4 NAA | 4.0±0.6 | 2.2±0.8 | 8 | 5.0±0.7 | 2.6±0.5 | 9 | 3.0±0.3 | 1.9±0.3 | 7 |
| 0.6 NAA | 2.5±0.4 | 2.7±0.5 | 14 | 5.0±0.4 | 5.2±1.1 | 11 | 3.1±0.3 | 3.2±0.7 | 11 |
| 0.3 BA+0.3 NAA | 4.2±0.3 | 2.8±0.2 | 4 | 4.7±0.4 | 1.9±0.3 | 5 | 4.8±0.5 | 2.5±0.5 | 3 |
| 0.3 BA+0.4 NAA | 4.9±0.5 | 2.4±0.1 | 5 | 4.4±1.4 | 1.6±0.4 | 6 | 2.9±0.3 | 1.6±0.3 | 4 |
| 0.3 BA+0.6 NAA | 3.2±0.4 | 1.5±0.2 | 16 | 6.3±0.5 | 4.7±0.7 | 12 | 2.9±0.2 | 2.6±0.4 | 13 |
| 0.4 BA+0.3 NAA | 4.8±0.4 | 3.2±0.9 | 3 | 6.4±1.4 | 2.0±0.3 | 7 | 3.2±0.5 | 1.3±0.2 | 9 |
| 0.4 BA+0.4 NAA | 3.6±0.5 | 2.1±0.2 | 6 | 5.4±0.6 | 1.7±0.3 | 8 | 5.2±0.5 | 2.1±0.4 | 8 |
| 0.4 BA+0.6 NAA | 4.8±0.4 | 3.3±0.8 | 10 | 5.6±0.4 | 3.9±0.4 | 13 | 4.0±0.8 | 2.7±0.5 | 12 |
| 0.6 BA+0.3 NAA | 5.5±0.9 | 3.1±0.5 | 11 | 5.4±0.6 | 2.3±0.2 | 16 | 5.3±0.6 | 3.6±0.2 | 15 |
| 0.6 BA+0.4 NAA | 3.9±0.4 | 2.1±0.4 | 13 | 5.8±0.4 | 2.8±0.2 | 14 | 4.4±0.4 | 2.8±0.3 | 14 |
| 0.6 BA+0.6 NAA | 3.6±0.5 | 1.7±0.3 | 15 | 5.4±0.8 | 2.4±0.3 | 15 | 3.5±0.4 | 2.4±0.4 | 16 |

* MS-control medium, without adding plant growth regulators

Table 2: The effects of plant growth regulators on callus formation from shoot tip explants of cucumber cultivars in MS medium

| | Calls formation (%) | | |
|---|---|---|---|
| PGR treatments | Telegraph 314 | Pepinex 69 | Rebella |
| PGR-free | 50 | 0 | 9 |
| BA | 87 | 87 | 97 |
| NAA | 90 | 93 | 92 |
| BA+NAA | 96 | 93 | 100 |

Table 3: The effects of plant growth regulators on extent of callus formation from shoot tip explants of cucumber cultivars in MS medium

| | Extent of callus formation | | |
|---|---|---|---|
| PGR treatments | Telegraph 314 | Pepinex 69 | Rebella |
| PGR-free | 0.4 | 0.0 | 0.5 |
| BA | 3.5 | 4.1 | 3.2 |
| NAA | 4.5 | 3.9 | 3.5 |
| BA+NAA | 4.7 | 4.5 | 3.6 |

Table 4: The effects of plant growth regulators on root induction from shoot tip explants of cucumber cultivars in MS medium

| | Roof formation (%) | | |
|---|---|---|---|
| PGR treatments | Telegraph 314 | Pepinex 69 | Rebella |
| PGR-free | 90 | 100 | 100 |
| BA | 55 | 77 | 79 |
| NAA | 55 | 71 | 75 |
| BA+NAA | 45 | 51 | 52 |

Table 5: The effects of plant growth regulators on extent of root induction from shoot tip explants of cucumber cultivars in MS medium

| | Extent of root formation | | |
|---|---|---|---|
| PGR treatments | Telegraph 314 | Pepinex 69 | Rebella |
| PGR-free | 2.3 | 2.4 | 2.1 |
| BA | 2.2 | 2.7 | 2.9 |
| NAA | 2.1 | 2.7 | 2.9 |
| BA+NAA | 1.8 | 2.0 | 1.2 |

(the values of different concentrations were pooled for each PGR treatment) on callus formation from shoot tip explants of cucumber cultivars were summarized in Table 2 and 3.

In addition, the shoots proliferated from the shoot tip explants also succeeded to root in each treatment. Hence, the effects of PGR treatments (the values of different concentrations were pooled for each PGR treatment) on root formation from shoot tip explants of cucumber cultivars were summarized in Table 4 and 5. Higher rooting frequency was obtained from the PGR-free medium compared to the PGR supplemented ones. On the other hand, the effects of PGR treatments on extent of root formation were cultivar depended. BA and NAA increased the root growth in cvs. Rebella and Pepinex 64, but had no effect in cv. Telegraph 314 when compared to PGR-free medium. Contrarily, the root development in each cultivar has been suppressed on the media containing combinations of BA and NAA. Nevertheless, almost all (85-90%) rooted plantlets were acclimated *ex vitro* and potted plants transferred to greenhouse conditions, where they grew well, did not show any morphological changes after 2 months of transplanting.

## DISCUSSION

The regeneration of cucumber plants via direct organogenesis from nodal and shoot tip explants are necessary in order to obtain plantlets with uniform growth characteristics of the mother plant for commercial mass

propagation. Previous works on *in vitro* propagation of cucumber from shoot tips indicate low shoot regeneration frequencies (Handley and Chambliss, 1979; Vasudevan *et al*., 2004). However, in the present study, it was possible to obtained 100% shoot regeneration response from shoot tip explants of each cucumber cultivar on either PGR containing media or even on the PGR-free ones (Table 1). Compared to the results of the previous research, high shoot frequency observed in the present study could be due to the genotypic differences. Mohiuddin *et al*. (1997) observed similar genotypic differences in shoot regeneration from cotyledon and hypocotyl explants of five cucumber cultivars. On the other hand, the shoot tip explants used by Vasudevan *et al*. (2004) were obtained from 5-day-old *in vitro* grown seedlings and cultured 1 week while the same explants were excised from 13-day-old seedlings and cultured 32 days in the current study. Therefore, the age of explants and the duration of culture might be the other factors which caused higher culture response in cucumber cultivars which were examined. Bhatia *et al*. (2004) have also stated that regenerative capacity of tomato increased with an increase in the age of the explants.

BA and NAA at different concentrations and combinations were tested for assessing optimum PGR treatment for maximum proliferation of cucumber from shoot tips. BA was found to be more effective than NAA on the proliferation. Considering all the parameters studied, the optimum treatment was 0.4 μM BA since it produced multiple shoots in the best quality. The fortification of BA at lower concentrations to the culture medium for multiple shoot induction from nodal explants of cucumber has also been reported by Ahmad and Anis (2005). The optimum shoot development was also obtained on the medium containing 0.3 μM NAA alone or in combination with 0.3 μM BA. The explants cultured with higher concentration of BA and NAA alone or in combination resulted low proliferation rate and/or shoot quality as reported by Sarowar *et al*. (2003) for *Cucurbita* interspecific hybrid cv. Shintosa. Rooting occurred in all the treatments but with different frequencies and the optimal response was observed on the PGR-free medium. Addition of BA and NAA to the culture media increased the extent of root formation, whilst the combination of both PGR inhibited the root development. Root formation on PGR free MS medium also reported in cucumber nodal culture by Konstas and Kintzios (2003).

It is concluded that the procedure described here appears to be adaptable for large clonal propagation of *Cucumis sativus* L. *in vitro* and could be used for reducing the high cost of hybrid seed production.

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