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Kinetin Is the Most Effective Cytokinin on Shoot Multiplication from Cucumber

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Abstract

In vitro shoot multiplication of cucumber (*Cucumis sativus*) was examined from the nodal explants of 10-day-old aseptic plantlets using Murashige and Skoog (MS) media supplemented with different concentration (0, 0.5, 1, 2, 3 mg L^{-1}) of cytokinins (6-Benzylaminopurine-BAP, Kinetin-Kn, Thidiazuron-TDZ, and Zeatin). Nodal explants of cucumber showed shoot induction and multiplication in response to all cytokinins tried. MS medium containing Kn was the most effective for inducing shoots from nodal explants of cucumber. The maximum rate of regeneration (83%), the highest number of obtained shoots (7.93 shoots/explant) and the longest shoots (3.61 cm) were obtained on MS medium fortified with 1 mg L^{-1} Kn. The lowest culture responses were recorded for media supplemented with either BAP or Zeatin. In conclusion, using Kn is strongly recommended than using Zeatin, TDZ and BAP to obtain the highest percentage of regeneration, the highest number of shoot/explant, and the highest shoot length for nodal explants of cucumber.

Keywords: cucumber, cytokinin, nodal segment, shoot multiplication

1. Introduction

Cucurbitaceae, commonly known as cucurbits, includes several economically important cultivated plants, such as watermelon (*Citrullus lanatus* (Thunb.), cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.) and squash (*Cucurbita* spp.) (Robinson & Decker-Walters, 1997). Cucurbitaceae has about 118 genera and 825 species that are distributed mainly in tropical and subtropical regions. Cucurbitaceae, believed to be domesticated in central Asia more than 3,000 years ago, is among the most important plant families as a source of vegetables (Harlan, 1971; Dagmar et al., 2010). The cucurbits are used as model plants for the study of vascular biology, since both xylem and phloem sap can be readily collected of long-distance signaling events (Xoconostle-Cázares et al., 1999; Lough & Lucas, 2006).

Cucumber (*C. sativus* L.) is a monoecious, annual, herbaceous and vining plant. Cultivated cucumbers, distributed throughout the world, are the fourth most important vegetable crop behind tomato, cabbage and onion (Tatlioglu, 1993). Cucumber fruits are edible and very much used as salad. Seeds are cooling, tonic, diuretic and anthelmintic. Flavone glycosides such as isovitexin, saponarin and various acylated flavone C-glycosides are present in the leaves of *Cucumis sativus* (Abou-Zaid et al., 2001). Recently, antiulcer 9-beta-methyl-19-norlanosta-5-ene type glycosides have been extracted from *Cucumis sativus* seeds (Gill & Bali, 2012).

Cucumber is extremely difficult to propagate vegetatively *in vivo*. Conventional breeding aiming to transfer desirable traits from wild species has not been successful (Esquinas-Alcazer & Gulick, 1983). Therefore, the development of *in vitro* micropropagation methods is very useful for its clonal multiplication (Deakin et al., 1971; Gaba et al., 2004). Tissue culture methods allow establishing cultures from a minimum amount of starting plant material (Klavina et al., 2006), with a lower chance of obtaining mutants comparing with conventional breeding.

Moreover, transplanting system of *in vitro*-derived plantlets is a suitable-alternative strategy for more efficient use of greenhouse and outdoor space, because seed germination and early growth of plants can be confined to a smaller nursery area (Vasudevan et al., 2001).

In vitro production of cucumber has been reported using various culture techniques. Variety of explants have been used as starting material for plant regeneration from cucumber, such as nodal segments (Ahmad & Anis, 2005), shoot tip (Vasudevan et al., 2001; Jafar & Nuray, 2007), cotyledons (Trulson & Shahin, 1986; Kim et al., 1988; Chee, 1990), hypocotyl explants (Rajasekaran et al., 1983; Ziv & Gadasi, 1986; Selvaraj et al., 2006), primary leaves (Chee & Tricoli, 1988; Malepszy & Nadolska-Orezyk, 1989; Seo et al., 2000), and petioles (Punja et al., 1990). Regeneration of cucumber is also possible through protoplast, leaf-derived callus (Abu-Romman et al., 2013), somatic embryogenesis (Chee, 1990), and cell suspension culture (Chee & Tricoli, 1988; Raharjo & Punja, 1992; Lou & Kako, 1994; Vengadesan et al., 2005; Ugandhar et al., 2011). Moreover, *in vitro* culture of cucumber has been recently used to evaluate cucumber physiological response to osmotic stress (Abu-Romman & Suwwan, 2011, 2012).

Cytokinin and auxin are the most common plant growth regulators used in i*n vitro* culture of plant tissues (Eudes et al., 2003). Cytokinins constitute a major class of plant growth regulator that is involved in a wide range of physiological processes (Davies, 1995). Cytokinins have been shown to induce in dark, a number of processes normally controlled by light, including amaranthin synthesis, chloroplast development, and differentiation of leaves and cotyledons (Chory, 1993).

The number of chemicals fitting the definition of cytokinins has grown to include a large array of natural and synthetic compounds derivatives, since the discovery of the first cytokinin, kinetin, by Miller and associates in 1955. Two types of cytokinins are well organized. The first one is adenine-type cytokinins represented by kinetin, zeatin, and 6-benzylaminopurine. The second type is phenylurea-type cytokinins represented by diphenylurea and thidiazuron (TDZ) (D. W. S. Mok & M. C. Mok, 2001). The chemistry of these two groups of cytokinins has been studied extensively. Natural cytokinins are classified chemically N6-substituted purine derivatives. Dihydrozeatin, Isopentenyladenine, and zeatin, are the predominant cytokinins found in higher plants (Letham, 1994). Glycosidic conjugates have major role in cytokinin transport, protection from degradation, and reversible and irreversible inactivation (Letham, 1994).

The biological functions of cytokinins in whole plants and tissues cultures have also been established (Mok et al., 1987; Mok, 1994). Moreover, the effect of endogenous cytokinins has been studied in many species (Auer, 1997). Cytokinins have a stimulatory or an inhibitory role in different developmental processes, such as control of apical dominance in the shoot, root growth and branching, leaf senescence, and chloroplast development (Mok, 1994). Several studies proved that cytokinin has a key role in *C. sativus* shoot induction (Ahmad & Anis, 2005; Mahmoud & Arash, 2014). However, Abu-Romman et al. (2013) found that callus induction frequency, callus growth rate and nature of callus in cucumber are significantly affected by the type and the concentration of the plant growth regulators, and are significantly higher when incorporating auxins in the medium compared to cytokinins. In this study, we examine the effect of four different cytokinins with different fold concentrations on percentage of regeneration, number of shoot/explant, and the length of shoots of nodal explant, to find out which form and concentration of cytokinin gives the maximum physiological response.

2. Materials and Methods

2.1 Establishment of Aseptic Seedlings

Seeds of cucumber (*Cucumis sativus* L. cv Beit Alpha) were prepared and sterilized according to Abu-Romman and Suwwan (2011 and 2012). Briefly, seeds were soaked in 70% (v/v) ethanol for 1 minute, and then surface sterilized by immersing in 1% sodium hypochlorite with 6 drops of Tween-20 per 100 ml for 20 minutes. Finally, the seeds were rinsed four times with sterile distilled water. The sterilized seeds were germinated on MS bioregulators-free medium. Nodal segments obtained from 10-day-old aseptic seedlings were used as explants.

2.2 Culture Media and Growth Conditions

MS medium (Murashige & Skoog, 1962) was used in all treatments. The basal medium for seed germination and shoot multiplications contained MS salts, 1% Bacto agar, 3% sucrose and 0.1 g L^{-1} myoinositol. The pH of the medium was adjusted to 5.7, and then was autoclaved at 121 ºC and 15 psi for 20 minutes. Seed germination and nodal cultures were maintained in a growth chamber at 22 ºC with 16 hr photoperiod using fluorescent light providing a PFD of 40 µmol m^{-2} s^{-1}.

2.3 Shoot Multiplication

Single node stem cuttings were taken from *in vitro* grown plantlets and inoculated on MS medium supplemented

individually with different concentrations (0, 0.5, 1, 2, 3 mg L^{-1}) of BAP, Kn, TDZ, and Zeatin. The frequency of regeneration, number of shoots per explant and shoot length were recorded after 4 weeks of culture.

2.4 Statistical Analysis

Treatments were arranged in a completely randomized design with 15 replicates. The data were analyzed statistically using the analysis of variance (ANOVA), and the significant differences between means were separated according to the least significant difference (LSD) at 0.05 level of probability using SAS program.

3. Results and Discussion

The treatment of the different types and concentrations of cytokinin shows different effect on percentage of regeneration, number of shoot/explant, and average shoot length.

However, in all the treatments, regardless of the different concentrations and the different types of cytokinin, increasing hormone concentration increases the physiological response initially until a saturation level has been reached. Increasing the hormone concentration after the "Saturation Level" gives an inhibitory physiological response; for example, when the nodal explant grown in four fold difference of BAP concentrations, the percentage of regeneration increases 10% by increasing the concentration of BAP from 0.5 mg L^{-1} to 1.0 mg L^{-1} (Table 1). However, this percent of increase of percentage of regeneration is not linear with fold increase of BAP concentration. Therefore, when 2.0 mg L^{-1} is used for nodal explant, the percentage of regeneration increases only by 4%. This illustrates the relationship of hormonal dose with physiological response. When hormonal concentration increases, physiological response increases significantly until the plant reaches the "Saturation Level", in which applying higher concentration of the hormone does not impact the plant to give the same rate of physiological response before reaching the saturation point. Therefore, applying higher concentration of the hormone after the plant reaches the saturation level will only give a decline in physiological response. This explains, in this study, the decrease in numbers measuring the physiological response with all the hormonal treatments using high hormone concentrations regardless of the type of cytokinin.

Table 1. Effect of BAP on shoot regeneration from nodal explants of *Cucumis sativus* after 4 weeks of culture. Means followed by different letters are significantly different according to LSD test at $P < 0.05$

BAP (mg L^{-1})	% Regeneration	No. of shoots/explant	Average shoot length (cm)
0	30	0.82 c	1.24 c
0.5	45	1.84 a	1.38 b
1.0	50	2.23 a	1.70 a
2.0	52	2.15 a	1.65 a
3.0	40	2.19 a	1.33 b

In Cucurbitaceae, media supplemented with cytokinins were reported critical for shoot regeneration (Sangeetha & Venkatachalam, 2014). In the present study, the overall maximum physiological response was obtained using Kn comparing using BAP, TDZ and Zeatin at the same concentration (Table 2). The second type of cytokinin that gives the overall second higher physiological response was TDZ (Table 3). Using BAP and Zeatin gives similar overall physiological response. However, using Zeatin gives higher number of shoot/explant and higher average shoot length than using BAP (Table 4), while using BAP gives slightly higher percentage of regeneration than using Zeatin.

Table 2. Effect of Kn on shoot regeneration from nodal explants of *Cucumis sativus* after 4 weeks of culture. Means followed by different letters are significantly different according to LSD test at $P < 0.05$

Kn (mg L^{-1})	% Regeneration	No. of shoots/explant	Average shoot length (cm)
0	30	0.82 c	1.24 c
0.5	52	4.85 b	2.52 b
1.0	83	7.93 a	3.61 a
2.0	80	5.71 b	3.08 ab
3.0	61	5.06 b	2.37 b

Table 3. Effect of TDZ on shoot regeneration from nodal explants of *Cucumis sativus* after 4 weeks of culture. Means followed by different letters are significantly different according to LSD test at $P < 0.05$

TDZ (mg L^{-1})	% Regeneration	No. of shoots/explant	Average shoot length (cm)
0	30	0.82 c	1.24 c
0.5	62	3.20 b	1.42 b
1.0	55	3.34 b	2.68 a
2.0	56	4.57 a	2.71 a
3.0	50	4.02 ab	2.46 a

Table 4. Effect of Zeatin on shoot regeneration from nodal explants of *Cucumis sativus* after 4 weeks of culture. Means followed by different letters are significantly different according to LSD test at $P < 0.05$

Zeatin (mg L^{-1})	% Regeneration	No. of shoots/explant	Average shoot length (cm)
0	30	0.82 c	1.24 c
0.5	37	1.54 b	1.90 a
1.0	50	2.87 a	1.75 a
2.0	45	1.60 b	1.73 a
3.0	40	1.62 b	1.38 b

The difference of physiological response of using Kn instead of the other types of cytokinin is big. For example, Kn gives more than two fold of increasing the number of shoots/explant than the other three types of cytokinin at concentration of 1.0 mg L^{-1} (Table 2). Even when using 2.0 mg L^{-1} concentration of TDZ, the number of shoots/explant using Kn, with a concentration of 1.0 mg L^{-1}, is more by 1.7 fold. This indicates how using Kn is favorable that using any of the other three types of cytokinin in order to obtain the highest number of shoots/explant. Similar conclusion applies regarding the average shoot length with less fold difference between the four different types of cytokinin. For example, more than two fold difference on the average shoot length (cm) is obtained using the same concentration of cytokinin of 1.0 mg/l, when using Kn instead of BAP. Less fold difference is obtained on average shoot length of using TDZ instead of Kn, but with two fold higher concentration of TDZ than Kn. In contrast to our results, BAP was reported in literature as the most effective cytokinin for shoot induction and multiplication in cucurbits (Ananthakrishnan et al., 2003; Saha et al., 2007; Ganasan & Huyop, 2010)

The percentage of regeneration using Kn is a at least a fold and half more than the percentage of regeneration using any of the other three types of cytokinin. After obtaining the highest physiological response, the percentage of regeneration is not inhibited much by increasing the concentration of cytokinin regardless of its type.

4. Conclusion

In conclusion, using Kn is strongly recommended than using Zeatin, TDZ and BAP to obtain the highest percentage of regeneration, the highest number of shoot/explant, and the highest shoot length for nodal explants of cucumber.

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