

Stimulatory Effects of Different Cytokinin on Direct Plant Regeneration from Cotyledon Explants in *Cucumis sativus* L.

Meng Zhang & Hongwen Cui

Horticulture College, Northwest Sci-Tech University of Agriculture & Forestry

Bingshan Wang & Shirong Jia

Biotechnology Institutes, Chinese Academy of Agriculture Sciences

In order to establish a regeneration system for gene transformation, the effect of four kinds of cytokinins (KT, BA, ZT and TDZ) were studied on direct shoot regeneration from cotyledons of 5-day-old seedlings of *Cucumis sativus* L. inbred line Jin431. ZT had a highest efficiency among the four cytokinins, and its regeneration frequency of 85% with multiple shoots may be useful for gene transformation. TDZ had the highest activation, and the optimal concentration of TDZ was considerably lower than that of the other three cytokinins. A new phenomenon, in which roots were induced as well as shoots by TDZ, was found.

Keywords: cucumber; cytokinin; organogenesis; cotyledon

Introduction: Cucumber (*Cucumis sativus* L.) is an important species of Cucurbitaceae and, with the exception of watermelon, is cultivated more than any other cucurbit in China. Recent attention has been directed towards gene transformation technology as a way of introducing heterogeneous genes into cucumber without the limits of cross incompatibility between species. There have been about 14 studies on cucumber gene transformation by the way of *Agrobacterium*-medium or microprojectile bombardment from 1986. Only a few of these studies have obtained transformants. Gene transformation systems depend on some form of plant regeneration technology. Most of the studies dealt with indirect plant regeneration. A high frequency of variation in regenerated plants occurs at the callus or somatic embryo stage (Malepszy & Nadolska-Orczyk, 1989). Some studies found that direct shoot regeneration (organogenesis) from explants in cucumber were free of morphological or physiological variation (Burza & Malepszy, 1995; Plader et al., 1998). A system suitable for transformation should ideally produce multiple shoots from numerous adventitious buds with minimal callus. This paper describes the effects of different cytokinins on organogenesis.

Materials and Methods: *Explant materials.* Seeds of *Cucumis sativus* L. inbred line Jin431 were surface-sterilized for 10 min using a dilution of commercial sodium hypochlorite having 3.5% active chlorine, then rinsed three times in sterile deionized water. The seeds were germinated on modified Murashige & Skoog (MS, 1962) basal medium supplemented with 3% sucrose and solidified with 0.75% agar. The pH was adjusted to 5.8 with 0.1N NaOH prior to sterilization at 121 C for 15 min. All plant material was incubated at 25 C with 16 hr photoperiod at 5.0 klx provided by cool white fluorescent lighting. Cotyledons from five-day-old seedlings were excised and cut transversely, and the basal segments without axillary buds were used for all experiments. Explants were placed with the abaxial side down on shoot induction media with the basal end just beneath the surface of the medium.

Methods: In the preliminary experiments, it was found that cytokinin was sufficient for shoot induction without auxins, while auxins can increase callus growth and decrease shoot development. In order to investigate the influence of different cytokinins on shoot induction and to select the optimal concentration, shoot induction media were prepared by modifying the MS medium above with the following concentrations of individual cytokinins: (1) kinetin (KT) concentrations at 0, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 mg l⁻¹, (2) 6-benzylaminopurine (BA) concentrations at: 0, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg l⁻¹, (3) zeatin (ZT) concentrations at: 0, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mg l⁻¹, (4) and thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5yl-urea, TDZ) concentrations at: 0, 0.0001, 0.001, 0.005, 0.01, 0.05, and 0.1 mg l⁻¹. (KT, BA and ZT were obtained from Sigma Co., while TDZ was synthesized by the Northwest Plant Institute, Chinese Academy of Sciences.)

Explants were cultured on shoot induction media under the same conditions as seedlings. There were 10 replicates per treatment with four explants per

replicate. Shoot differentiation and induction frequencies were investigated at the 20th day after induction. Statistical analysis was conducted according to a single-factor design.

Results: Protuberances could be found at the basal region of cotyledons after 7-8 days induction when cytokinin concentrations were suitable, and little buds could be found after two weeks. These buds could develop to 1-2 cm in size after 3 weeks (Fig.1).

The effects of a variety of different cotyledons on shoot induction, and statistical analyses of KT, BA and ZT concentrations are shown in Table 1, while the results of shoot induction frequencies and statistical analysis of TDZ are shown in Table 2, since the range of TDZ concentrations was much lower than the other three. The results of the four kinds of cytokinins exhibited the same tendency in that shoot induction frequency was low and the callus grew slowly in media with low cytokinin concentrations, shoot induction became optimal and the callus grew moderately with increasing cytokinin concentrations, and shoot induction then decreased sharply and while callus growth was greatest with the further increases in cytokinin concentration, although the callus grew little at the highest concentration of cytokinin. The cytokinins KT, BA and ZT could induce multiple shoots at the basal region of cotyledons in the media with each cytokinin's optimal concentration. The number of shoots induced per explant was usually more than 10, and 25-30 at maximum. The cytokinin TDZ induced fewer shoots at the base of the cotyledon than the other three cytokinins, with fewer than 3 shoot developed per explant. There was a new phenomenon in that roots were induced as well as shoots in the shoot induction medium with a TDZ concentration of 0.0001 to 0.01 mg l⁻¹ (Fig. 2). The root induction frequency increased with decreasing concentration.

The optimal concentration of KT was 5.0 mg l⁻¹, of BA was 0.5 mg l⁻¹, of ZT was 1.0 mg l⁻¹, and of TDZ was 0.005 mg l⁻¹. The cytokinin TDZ had a higher activation on shoot induction than the other three, and the optimal concentration of TDZ was considerably lower. ZT had the highest efficiency (85%) of shoot induction of the selected inbred line. The concentration range of cytokinins on shoot induction differed from each other. TDZ could

induce shoots at concentration of 0.00001 to 0.05 mg l⁻¹ and ZT had a relative high effective at concentration of 0.2 to 5.0 mg l⁻¹, while KT and BA had a relative narrow range of concentration on shoot induction.

Discussion: Wehner and Locy (1981) studied the direct regeneration with cotyledon and hypocotyl from 7-day-old seedlings as explants on the MS medium containing 1.0mg/l BA and 1.0 mg/l⁻¹ NAA. Eighty-five cultivars were tested and just 28 cultivars developed shoots with an average shoot induction frequency of 5.5%. That is likely due to the addition of NAA and explants from elder seedling. In order to establish the cucumber gene transformation system, Gambley and Dodd (1990,1991) studied successfully the shoot direct regeneration with three kinds of cytokinins KT, BA and 2-ip (N⁶-(2-isopentenyl)adenine). They found that concentration (4 mg l⁻¹ or less) of the cytokinins KT, BA and 2-ip with or without low concentration of auxin, were all effective in producing multiple adventitious buds at the base of cotyledons from 5-7 -day-old seedling. BA had a best efficiency in the three kinds of cytokinins. In this studied, the similar results were obtained. We found that the auxin restrained the shoot development even at low concentration in the preliminary experiments (data not showed), and the ZT had a highest efficiency on inducing shoots of the selected inbred line among the four kinds of cytokinins. So this multiple adventitious shoots regeneration technique should be useful for transformation studies in cucumber. We had got some of chitinase/ β-1, 3-glucuronidase genes and glucose oxidase gene transformants by this direct organogenesis technique.

On the other hand, we observed a new phenomenon that roots were induced as well as shoots were induced. That may be due to the higher endogenesis auxins compared with the absolute amount of TDZ.

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Table 1. Shoot induction efficiency of three kinds of cytokinins

cytokinins	Concentration (mg/l)							
	0	0.2	0.5	1.0	2.0	5.0	10.0	20.0
KT	0dC		5cdBC	20bB	20bB	50aA	15bcB	0dC
BA	0dC	20cC	79aA	70abAB	55bB	8dC	0dC	
ZT	0cC	45bB	60bAB	85aA	50bB	39bB	0cC	

Notes: a,b,c and d indicate significance at the 0.05 level.
A, B and C indicate significance at the 0.01 level

Table 2. Shoot induction efficiency of different concentration of TDZ

Concentration (mg/l)	Shoot induction (%)	significance level (0.05)	significance level (0.01)
0.005	60	a	A
0.01	35	b	B
0.05	25	bc	BC
0.001	20	c	BC
0.0001	5	d	CD
0.1	0	d	D
0	0	d	D

Notes: a,b,c and d indicate significance at the 0.05 level.
A, B and C indicate significance at the 0.01 level



Fig. 1 Shoots induced by cytokinin ZT.



Fig. 2 Shoot and roots induced by cytokinin TDZ.