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Effects of Various Explants and Hormone Combinations on in *vitro* Regeneration in Cucumber

Yanhua Li, Yongdong Sun*, Weirong Luo, Lei Ni

School of Horticulture and Landscape Architecture, Henan Institute of Science and Technology, Xinxiang, Henan, 45300, P. R. China,
sunyd2001@163.com

Effects of various explants and hormone combinations on *in vitro* regeneration in cucumber (*Cucumis sativus*) were studied using Jinyou No. 1. The results showed that explant types had a greater impact on the regeneration rate and number of regenerated buds in cucumber. When using 1–2-d-old sterilized cotyledons as explants, Murashige and Skoog (MS) supplemented with 6-benzylaminopurine (6-BA; 3.0 mg L^{-1}), kinetin (KT; 1.0 mg L^{-1}), abscisic acid (ABA; 1.0 mg L^{-1}), and $AgNO_3$ (2.0 mg L^{-1}) was the best bud induction medium, with an adventitious bud regeneration rate of 73.3%, and 1.00 regenerated bud per explant. When using 5–6-d-old sterilized cotyledonary nodes as explants, MS supplemented with 6-BA (2.0 mg L^{-1}) and $AgNO_3$ (2.0 mg L^{-1}) was the best, with an adventitious bud regeneration rate of 90.70% and 4.38 regenerated buds per explant. In this study, cotyledonary nodes were more suitable than cotyledons for the establishment of the *in vitro* regeneration system in cucumber, and MS containing 6-BA (2.0 mg L^{-1}) was the best bud induction medium for cotyledonary nodes, which may have valuable applications for an efficient in vitro regeneration protocol.

1. Introduction

Cucumber is an important member of the family Cucurbitaceae and is commonly used as a fruit and vegetable in regular diets. Cucumber plays an extremely important role in the vegetable supply and national economy (Wang *et al*., 2013). However, a low fruit set rate is a serious problem in agricultural production, especially under protected cultivation conditions because of low temperature and low light resulting from rain and snow or fog haze weather, which seriously affects the high yield and good quality of cucumber. Recent developments in plant *in vitro* culture techniques and genetic engineering have enabled the establishment of a highly efficient regeneration system and genetic engineering techniques for germplasm innovations, and these methods have improved the yield and quality of crops (Kielkiewicz *et al*., 2013). Therefore, it is imperative to use genetic transformation tools for the improvement of quality traits in cucumber. For the development of genetically transformed plants, an efficient in vitro regeneration protocol is required.
In vitro regeneration of cucumber has been achieved by using different culture techniques (Malepszy 1988; Chee, 1990; Lou and Kako, 1994Mohiuddin *et al*., 1997; Selvaraj *et al*., 2007). Colijn-hooymans *et al*. (1994) found that seedling age has a high impact on cucumber regeneration, and 3–5-d-old cotyledon explants have the highest adventitious bud regeneration rate. Seo *et al*. (2000) established a shoot regeneration using leaf explants of cucumber as explants. Selvaraj *et al*. (2007) established a shoot regeneration from cotyledon explants of cucumber *via* organogenesis. Fan *et al*. (2008) used cotyledonary nodes as explants and optimized the seedling status, methods for explant dissection and inoculation, and genotypes to improve the cucumber regeneration system. Zhang *et al*. (2010) established a cucumber cotyledon regeneration system based on genotypes and hormones. Shukla *et al*. (2014) established *in vitro* shoot regeneration progress in *Cucumis sativus* by inhibition of endogenous auxin. There has been great progress in cucumber tissue culture techniques, however, issues such as low regeneration rate and low reproducibility still remain, seriously affecting the application of plant genetic engineering techniques to the molecular genetic improvement of cucumber. In the present study, effects of various explants and hormone combinations on cucumber *in vitro* regeneration were discussed, and the best bud induction medium for cotyledonary nodes were found, which

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may have valuable applications for an efficient in vitro regeneration protocol and genetic engineering studies in cucumber breeding.

2. Materials and methods

2.1 Plant material

Cucumber seeds “Jinyou No. 1” were purchased from the Tianjin Kernel Cucumber Research Institute.

2.2 Preparation of sterile seedlings

2.2.1 Preparation of cotyledons from sterile seedlings

Cucumber seeds of uniform size were selected and soaked in 55°C water for 5–20 min until the seed coat softened. The seed coat was removed from the wider side of the seed. The embryo without the seed coat was placed on a clean bench and sterilized with 70% alcohol for 1 min, followed by 0.1% mercuric chloride for 10 min, with gentle shaking. Embryos were rinsed with sterile water 3–5 times and inoculated onto the Murashige and Skoog (MS) medium. The MS medium (pH 5.8) consisted of 7 g/l agar and 30 g/l sucrose. The culture conditions were 25°C, with a light intensity of 2500 lx, and a photoperiod of 16 h/d.

2.2.2 Preparation of cotyledonary nodes from sterile seedlings

Cucumber seeds of uniform size were selected and washed with running water for 4–5 h. Seeds were placed on a clean bench and sterilized with 70% alcohol for 1 min, followed by 0.1% mercuric chloride for 20 min with gentle shaking. Seeds were washed with sterile water 5–6 times, and inoculated onto the MS medium and cultured in the dark at 28°C 24 h, and then moved to the light conditions described above.

2.3 Induction of adventitious buds

2.3.1 Induction of adventitious buds in cotyledons

Sterilized cucumber seedlings with cotyledons grown for 1–2 d but not yet fully expanded were selected. Both cotyledons were cut close to the growing point. The upper parts (1/2–1/3) of the cotyledons were removed and the central part was retained. Each cotyledon was cut into 4–6 pieces and placed upside down on the MS bud induction medium containing various concentrations of 6-benzylaminopurine (6-BA; 1.0, 2.0, and 3.0 mg L^{-1}), kinetin (KT; 0, 0.5, 1.0, and 1.5 mg L^{-1}), abscisic acid (ABA; 0, 0.5, and 1.0 mg L^{-1}), and $AgNO_3$ (2.0 mg L^{-1}). The culture was grown in the dark at 28°C for 48 h and then switched to light conditions. The number of adventitious buds was measured after 20 d.

2.3.2 Induction of adventitious buds in cotyledonary nodes

5–6-d-old sterilized cucumber seedlings with cotyledons that were not fully expanded were selected. The growing point and the upper part (1/2–2/3) of the both cotyledons were removed. The hypocotyl was dissected longitudinally, and a 2-mm segment of hypocotyl was retained. The cotyledonary nodes were inoculated on the MS bud induction medium.

2.3.3 Calculation of the regeneration rate and the number of regenerated buds

Regeneration rate (%) = (number of explants with regenerated adventitious buds / total number of explants) × 100.

The number of regenerated buds per explant = the total number of adventitious buds / the total number of explants with adventitious buds.

2.4 Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Analysis of variance (ANOVA) was followed by Tukey’s pair wise comparison tests, at a level of $P<0.05$, in order to determine the significant differences between means.

3. Results

3.1 Effects of various combinations and concentrations of hormones on *in vitro* regeneration from cucumber cotyledons

Induction of adventitious buds was closely related to the combinations and concentrations of hormones (Table 1). For the same concentration of 6-BA, different concentrations of KT and ABA resulted in different trends in adventitious bud regeneration rate and the number of regenerated buds among explants. In C_8 medium, the adventitious bud regeneration rate was 74.70%, significantly higher than that of other media, and the number of regenerated buds was 0.76. The adventitious bud regeneration rate using the C_9 medium was 73.30%. However, it resulted in the highest number of buds, 1.00 per explant. Therefore, the C_9 medium (MS + 3.0 mg L^{-1} 6-BA + 1.0 mg L^{-1} KZ + 1.0 mg L^{-1}ABA + 2.0 mg $L^{-1}AgNO_3$) was better to induce adventitious buds from cucumber cotyledons.

Table 1. Effects of various combinations and concentrations of hormones on in vitro regeneration from cucumber cotyledons and cotyledonary nodes

Medium	6-BA (mg L^{-1})	KT (mg L^{-1})	ABA (mg L^{-1})	Number of regenerated buds per explant		Regeneration rate (%)	
				cotyledons	cotyledonary nodes	cotyledons	cotyledonary nodes
A_1	1.0	0	0	0.24 cde	1.40 abc	8.60 fg	65.40 abc
A_2	1.0	0	0.5	0.23 cde	1.11 bc	54.40 abcde	57.10 abc
A_3	1.0	0	1.0	0.24 cde	0.60 bc	15.10 fg	25.80 cd
A_4	1.0	0.5	0	0 e	1.37 abc	18.10 efg	81.90 ab
A_5	1.0	0.5	0.5	0.38 bcde	1.40 abc	46.40 abcdef	58.10 abc
A_6	1.0	0.5	1.0	0.41 bcde	1.24 bc	43.40 abcdef	50.90 abcd
A_7	1.0	1.0	0	0.22 cde	1.28 abc	45.50 abcdef	54.60 abcd
A_8	1.0	1.0	0.5	0.49 bcd	1.99 abc	45.80 abcdef	86.00 ab
A_9	1.0	1.0	1.0	0.48 bcd	0.60 bc	43.30 abcdefg	35.00 cd
A_{10}	1.0	1.5	0	0 e	0.91 bc	8.40 fg	38.40 bcd
A_{11}	1.0	1.5	0.5	0.46 bcd	1.20 bc	44.10 abcdef	55.00 abc
A_{12}	1.0	1.5	1.0	0.45 bcd	0.76 bc	55.20 abcde	50.90 abcd
B_1	2.0	0	0	0.31 cde	4.38 a	14.00 fg	90.70 ab
B_2	2.0	0	0.5	0.32 cde	0.82 bc	29.40 cdefg	44.10 bcd
B_3	2.0	0	1.0	0.28 cde	0.54 bc	23.30 defg	43.00 abcd
B_4	2.0	0.5	0	0 e	1.24 bc	0 g	63.00 abc
B_5	2.0	0.5	0.5	0.61 abc	1.49 abc	56.80 cdefg	63.40 abc
B_6	2.0	0.5	1.0	0.41 bcde	2.63 abc	38.30 abcdefg	52.40 abcd
B_7	2.0	1.0	0	0.08 de	1.89 abc	8.90 fg	68.20 abc
B_8	2.0	1.0	0.5	0.22 cde	2.21 abc	41.50 abcdefg	59.30 abc
B_9	2.0	1.0	1.0	0.29cde	2.36 abc	29.70 cdefg	59.80 abc
B_{10}	2.0	1.5	0	0 e	3.07 ab	11.70 fg	88.30 ab
B_{11}	2.0	1.5	0.5	0.24 cde	1.06 bc	35.60 abcdefg	40.60 bcd
B_{12}	2.0	1.5	1.0	0.44 bcde	1.51 abc	34.40 abcdefg	45.60 abcd
C_1	3.0	0	0	0 e	2.44 abc	0 g	64.20 abc
C_2	3.0	0	0.5	0.17 cde	1.86 abc	57.10 abcde	86.20 ab
C_3	3.0	0	1.0	0 e	1.46 abc	11.50 fg	66.50 abc
C_4	3.0	0.5	0	0 e	3.30 ab	14.80 fg	96.20 a
C_5	3.0	0.5	0.5	0.14 de	1.89 abc	62.80 abcd	75.80 abc
C_6	3.0	0.5	1.0	0.33 cde	0.89 bc	34.80 bcdef	38.60 d
C_7	3.0	1.0	0	0.36 bcde	3.28 ab	8.80 fg	77.20 abc
C_8	3.0	1.0	0.5	0.76 e	1.72 abc	74.70 ab	71.80 abc
C_9	3.0	1.0	1.0	1.00 a	2.10 abc	73.30 a	68.70 abc
C_{10}	3.0	1.5	0	0.15 de	3.29 ab	0 g	90.20 ab
C_{11}	3.0	1.5	0.5	0.27 cde	2.89 abc	42.00 abcdef	68.50 abcd
C_{12}	3.0	1.5	1.0	0.25 cde	2.50 abc	64.00 abc	51.90 abcd
C_{13}	0	0	0	0 e	0 c	0 g	0 d

Note: Lower case letters indicate significant differences at $p < 0.05$.

3.2 Effects of various combinations and concentrations of hormones on the *in vitro* regeneration from cucumber cotyledonary nodes.

As shown in Table 1, the regeneration rate and the number of regenerated buds varied markedly using cotyledonary nodes as explants, depending on the combinations and concentrations of hormones. In the control C_{13} medium, the growth point of the cotyledonary node did not bulge, and the number of adventitious roots grew substantially with increasing culture time. The adventitious bud regeneration rate in the B_1 medium was relatively high at 90.70%. The B_1 medium also showed the highest number of regenerated buds, 4.38. The C_4 medium had the highest regeneration rate of 96.20%, and the number of generated buds was 3.30. In the C_{10} medium, the regeneration rate was 90.20% and the number of regenerated buds was 3.29. Therefore, the B_1 medium (MS + 2.0 mg L^{-1} 6-BA + 2.0 mg $L^{-1}AgNO_3$) was the best bud induction medium for cotyledonary nodes in this study.

3.3 Effects of explant types on *in vitro* cucumber regeneration

The explant types greatly affected cucumber regeneration (Table 1). In the C_9 medium, the adventitious bud regeneration rate and the number of regenerated buds of cotyledon were 73.30% and 1.00, respectively, whereas those of the cotyledonary node were 68.70% and 2.10, respectively. In the B_1 medium, the regeneration rate and the number of regenerated buds of the cotyledon were 14.00% and 0.31, respectively, whereas those of the cotyledonary node were 90.70% and 4.38, respectively. Thus, the number of regenerated buds for the cotyledonary nodes was much higher than that observed for the cotyledons under the same genotype and culture conditions, suggesting that cotyledonary nodes are more suitable for the establishment of the cucumber regeneration system.

3.4 Effects of 6-BA on *in vitro* regeneration using cotyledonary nodes

In medium lacing KT and ABA and containing 2.0 mg L^{-1} $AgNO_3$, different concentrations of 6-BA caused significant differences in the regeneration rate and the number of regenerated buds using cotyledonary nodes (Table 2). A 6-BA concentration of 2.0 mg L^{-1} resulted in the highest regeneration rate (90.70%) and the highest number of regenerated buds (4.38) for the cotyledonary nodes.

Table 2: Effects of 6-BA on in vitro regeneration using cotyledonary nodes

6-BA (mg L^{-1})	KT (mg L^{-1})	ABA (mg L^{-1})	Number of regenerated buds per explant	Regeneration rate (%)
0	0	0	0 c	0 c
1.0	0	0	1.40 bc	65.40 ab
2.0	0	0	4.38 a	90.70 a
3.0	0	0	2.44 ab	64.20 ab

Note: Lower case letters indicate significant differences at $p < 0.05$.

3.5 Effects of ABA on *in vitro* regeneration using cotyledonary nodes

In medium lacking KT but containing 6-BA (2.0 mg L^{-1}) and $AgNO_3$ (2.0 mg L^{-1}), the regeneration rate and number of regenerated buds of the cotyledonary nodes showed a decreasing trend with increasing concentrations of ABA (Table 3). For an ABA concentration of 0 mg L^{-1}, the highest regeneration rate and the maximum number of regenerated buds were obtained, and these differed significantly from those of other treatment concentrations.

Table 3: Effects of ABA on in vitro regeneration using cotyledonary nodes

6-BA (mg L^{-1})	KT (mg L^{-1})	ABA (mg L^{-1})	Number of regenerated buds per explant	Regeneration rate (%)
2.0	0	0	4.38 a	90.70 a
2.0	0	0.5	0.82 b	44.10 b
2.0	0	1.0	0.54 b	45.00 b

Note: Lower case letters indicate significant differences at $p < 0.05$.

3.6 Effects of KT on *in vitro* regeneration using cotyledonary nodes

As shown in Table 4, for medium lacking ABA but containing 6-BA (2.0 mg L^{-1}) and $AgNO_3$ (2.0 mg L^{-1}), for increasing KT concentrations, both the regeneration rate and the number of regenerated buds using the cotyledonary nodes first declined and then rose. For a KT concentration of 0 mg L^{-1}, both the regeneration rate

(90.70%) and the number of regenerated buds (4.38) were maximal. For a KT concentration of 0.5 mg L^{-1}, both the regeneration rate (63.00%) and the number of regenerated buds (1.24) were the lowest.

Table 4: Effects of KT on in vitro regeneration using cotyledonary nodes

6-BA (mg L^{-1})	ABA (mg L^{-1})	KT (mg L^{-1})	Number of regenerated buds per explant	Regeneration rate (%)
2.0	0	0	4.38 a	90.70 a
2.0	0	0.5	1.24 b	63.00 b
2.0	0	1.0	1.89 b	68.20 b
2.0	0	1.5	3.07 a	88.30 a

Note: Lower case letters indicate significant differences at $p < 0.05$.

4. Discussion

MS medium is often used as the basal medium for *in vitro* cucumber cultures. Supplementation with different types and concentrations of hormones results in regenerated plantlets. Hormone types, combinations, and concentrations used are generally selected based on genotype, regeneration method, and explant types (Kim *et al.* 1988). Optimal hormone types and concentrations are essential for a high frequency *in vitro* regeneration system. This study compared the effects of various combinations and concentrations of 6-BA, ABA, and KT on adventitious bud induction using cucumber cotyledons and cotyledonary nodes. We found that the number of regenerated buds for the cotyledonary nodes was much higher than that observed for the cotyledons under the same genotype and culture conditions, suggesting that cotyledonary nodes are more suitable for the establishment of the cucumber regeneration system. The addition of only 2.0 mg L^{-1} 6-BA resulted in the growth and expansion of cucumber cotyledonary nodes, with the highest adventitious bud regeneration rate and the most regenerated buds. These results indicate that 6-BA is an essential hormone for *in vitro* regeneration, ABA and KT had little effect on the differentiation of adventitious buds using cucumber cotyledonary nodes. Wang *et al.* (2006) also suggested that 6-BA is an essential hormone for *in vitro* regeneration using cucumber cotyledonary nodes as explant, but the best 6-BA concentration was 1.5 mg L^{-1}.

5. Conclusions

In this paper, effects of various explants and hormone combinations on the regeneration rates and number of regenerated buds in cucumber were examined, and the best bud induction medium for cotyledonary nodes were found, which laid the foundation for an efficient in vitro regeneration protocol and genetic engineering studies.

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