

EFFECTS OF EXOGENOUS PLANT GROWTH REGULATOR ON *IN VITRO* REGENERATION OF COTYLEDONAR EXPLANTS IN PEPPER

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Abstract. Regenerated plants were obtained from cotyledon explants of eight pepper varieties (*Capsicum annuum* L.) cultured on Murashige and Skoog (MS) basal medium supplemented with different plant growth regulator (PGRs) by four-step culture procedure including shoot induction, shoot elongation, shoot rooting and transplanting. Nicotinic acid is important for plant growth which can promote explants differentiation. AgNO₃ significantly increased the frequency of shoot induction in different pepper varieties. Gibberellic acid (GA₃) was the key factor in shoot elongation. The elongated shoots cultured on MS basal medium with indole-3-butyric acid (IBA) were easier to root than that on MS basal medium. Plantlets were transplanted to soil and acclimatized in the greenhouse showing normal development. By the four-step procedures stated above, a plant regeneration system was established.

Key words: *Capsicum annuum* L., cotyledon, *in vitro*, plant regeneration

INTRODUCTION

The regeneration from tissue culture serves the following main purpose: micropropagation of elite plants, fixation of hybridization vigor, preservation and application of variants, and genetic transformation (Dabauza *et al.*, 2001). Dicotyledoneous species differ widely in their organogenesis potential and amenability to genetic transformation. Genetic engineering of dicotyledoneous plants has been limited due to difficulties associated with efficient *in vitro* plant regeneration (Pozueta-Romero J. *et al.*, 2001).

Tissue culture technique in pepper, one of the most important vegetable crops in the world, lag behind most other vegetable crops, mainly due to its recalcitrance to regeneration. With the exception of a few procedures for adventitious shoot regeneration, regeneration of pepper has been obtained via protoplast, hypocotyls, cotyledons, young leaves, direct somatic embryogenesis and shoot bud organogenesis from seedling explants by culturing in MS basal medium supplemented with exogenous growth regulators (Díaz *et al.*, 1988).

However, these procedures failed or had to be modified when they were used to regenerate plants from other pepper varieties in other laboratories. Thus, the strong influence is pepper variety. Moreover low differentiation frequency, difficulty in shoot elongation, and low repeatability are main barriers to the development of pepper gene engineering (Dabauza M. *et al.*, 2001).

Both roots and cotyledons of young seedlings actively produce PGRs involved in the control of organogenesis (Hicks, 1994), so most techniques for regeneration depend on the use of PGRs in complex adapted to each particular situation. An exact combination of PGRs had to be empirically determined for different cultivars (Manoharan *et al.*, 1998). Therefore, there are still few reports in the refereed literature dealing with the Chinese pepper varieties. This observation prompted our hypothesis that the balance of PGRs produced by these organs might induce *de novo* regeneration of meristems. This report describes the development of a simple and efficient procedure for plant regeneration, in a PGRs medium, applicable to different cultivars of pepper. Consequently, a more efficient, reliable, simple, rapid and universal methods for pepper regeneration system was obtained to break through the limit of tissue culture to the development of gene transformation.

MATERIALS AND METHODS

Plant Materials, Culture Media and Experiment Conditions

Seeds of pepper varieties: Xiangjiao No.11, Ganjiao No.1, Zhongjiao No.7, Atlantic, Zhudachang, Jingyanlajiao No.3, Chaotianjiao and Tianza No.7 were used in the experiment.

Seeds were surface sterilized in 70 % (v/v) ethanol for 30 s, soaked in 0.1 % (w/v) HgCl₂ for 6 min, and then rinsed three times with sterile distilled water. The seeds were germinated on half strength MS basal medium (Murashige *et al.*, 1962) in 16 hours photoperiod per day with temperature set at 25°C.

Cotyledon explants

Cotyledons of seedlings 6-8 d after germination without petiole and apical parts were cut into small parts of 0.25 cm². Explants were placed with abaxial side on the culture medium supplemented with 6-benzyladenine (6-BA) (5.0 mg L⁻¹), indole-3-acetic acid (IAA) (1.0 mg L⁻¹), 2,4-D(0.5 mg L⁻¹) and AgNO₃(2.0mg L⁻¹, 4.0mg L⁻¹, 6.0mg L⁻¹, 8.0mg L⁻¹, 10.0mg L⁻¹), Nicotinic acid (0.5 mg L⁻¹, 1.0 mg L⁻¹nicotinic acid) for shoot induction with subculture at two weeks to the same medium.

Explants with buds and shoots were transferred to shoot elongation medium (MS medium+6.0 g L⁻¹ agar+30.0 g L⁻¹ sucrose+3.0 mg L⁻¹ BA) supplemented with IAA(0.5 mg L⁻¹, 1.0 mg L⁻¹) and GA₃ (1.0 mg L⁻¹, 2.0 mg L⁻¹) with a subculture at two weeks to the same medium. The culture media were adjusted to pH 5.7 and solidified with 0.6% agar before autoclaving at 121 °C for 15 min.

Embryonic cotyledon was cultured in 9 cm dishes with 25 ml of medium. Incubation was performed in a tissue culture chamber at 26 ± 1°C under 16 h photoperiod. At least 20 explants were used per treatment and all experiments were repeated three times. The percentages of explants with buds and shoots (2–8 mm length) were recorded after 30 days of

culture on shoot induction medium. The number of elongated shoots (longer than 5 mm) per explant was recorded after 30 days on elongation medium.

To test their rooting capacity, elongated shoots were excised and transferred to different media: MS either without plant growth regulators or with IBA. Plants with roots were transferred during three weeks, after washing off the agar with deionized water, to pots with a mixture of substrate and perlite (5:1). The regenerated plantlets were acclimatized for 48 hours before they were transferred. The plants were grown in a green house, developing into normal plants and bearing normal fruits.

RESULTS AND DISCUSSIONS

Effect of BA, IAA and 2, 4-D on Shoot Induction

BA, IAA and 2, 4-D were used as plant growth regulators in the experiment. Pepper explants could produce calli on all media containing plant growth regulators, but the morpha of calli was very different.

Cotyledon explants began to expand in general 5-7 d after they were inoculated, and it could be observed that white or green calli were produced at the wounds of explants. The media supplemented with 2,4-D significantly promoted the formation of calli. However, the calli were yellow and slack which could not differentiate to shoot (Figure 1).

Hard and dense calli could be obtained only with BA as plant growth regulators which resulted in no differentiation. It could have strong influence on shoot induction that IAA was added to BA. Dense tumor-like white or green calli formed cultivated on MS basal medium supplemented with BA and IAA (Figure 2). And clusters of shoots were formed cultivated for four weeks with high differentiation frequency (Table 1). It is recommended that BA added with IAA was used to induce shoot differentiation.

Table 1

Effect of 2,4-D and IAA on shoot induction (%)

Variant	Cultivars							
	Xiangjiao No.11	Ganjiao No.1	Atlantic	Zhongjiao No.7	Zhudachang	Tianza No.7	Jingyanlajiao No.3	Chaotianjiao
BA+2,4-D	0	0	0	0	0	0	0	0
BA+IAA	13.7±0.6	14.8±0.5	71.2±2.1	34.6±1.2	3.1±0.1	51.4±2.2	20.1±0.7	15.3±0.7

Note: MS medium+6.0 g L⁻¹ agar +30.0 g L⁻¹ sucrose+5.0 mg L⁻¹ 6-BA+0.5 mg L⁻¹ 2,4-D, MS medium+6.0 g L⁻¹ agar +30.0 g L⁻¹ sucrose +5.0 mg L⁻¹ 6-BA+1.0 mg L⁻¹ IAA; 20 explants for per plate, 2 dishes for per replication.



Figure 1.
Explants on the medium containing IAA

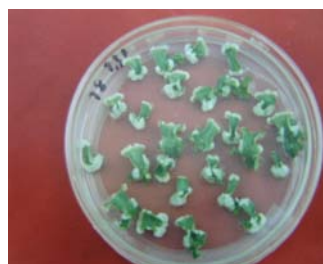


Figure 2.
Explants on the medium containing BA and IAA

Effect of Nicotinic Acid on explants differentiation of different pepper varieties

Nicotinic acid could promote shoot induction. When the media were supplemented with different concentration of Nicotinic acid, the rate of explants differentiation of eight pepper increased, for example Zhongjiao No.7, after adding 0.1 mg L^{-1} , 0.5 mg L^{-1} , 1.0 mg L^{-1} nicotinic acid in the medium, the rate increased from 34.6 % to 55.0 % , 96.7 % , 88.2 % separately. The rates of shoot induction were raised to various extents in other cultivars after nicotinic acid was added to the shoot induction medium.

Table 2 shows that 0.5 mg L^{-1} nicotinic acid was optimum for shoot induction with an average frequency of 61.8 %.

Table2

Effect of nicotinic acid on explants differentiation of different pepper varieties (%)

Cultivars	Media			
	A	B	C	D
Xiangjiao No.11	13.7±0.5	30.0±1.2	27.9±0.8	20.5±0.7
Ganjiao No.1	14.8±0.4	28.0±0.6	34.5±0.8	30.7±0.9
Atlantic	71.2±1.3	81.5±3.7	93.3±2.6	86.5±3.2
Zhongjiao No.7	34.6±1.2	60.0±2.1	80.7±3.8	72.2±2.9
Zhudachanglajiao	3.1±0.1	4.0±0.1	5.0±0.1	8.8±0.2
Tianza No.7	51.4±0.9	72.5±2.7	90.1±3.9	78.5±4.1
Jinyanlajiao	20.1±0.7	42.5±1.6	72.0±3.8	37.5±1.2
Chaotianjiao	15.3±0.4	63.0±2.8	83.3±3.2	56.8±1.3
Average	31.6	47.7	60.8	48.9

Note: A: MS medium+6.0 g L⁻¹ agar +30.0 g L⁻¹ sucrose +5.0 mg L⁻¹ BA+1.0 mg L⁻¹ IAA; B: A+0.1 mg L⁻¹ nicotinic acid; C: A+0.5 mg L⁻¹ nicotinic acid; D: A+1.0 mg L⁻¹ nicotinic acid

Effect of AgNO₃ on explants differentiation of different pepper varieties

The frequencies of explants differentiation could be dramatically increased with an average of 65.6 % in the medium containing BA and IAA supplemented with 2.0 mg L^{-1} AgNO₃ (Table 3). In all cultivars, the frequency of explants differentiation is highest on the medium with 4.0 mg L^{-1} AgNO₃. So AgNO₃ should be chosen as a chemical addition to promote shoot–bud induction.

Table 3

Effect of AgNO₃ on explants differentiation of different pepper varieties (%)

Cultivars	AgNO ₃ concentration (mg.L ⁻¹)					
	0	2.0	4.0	6.0	8.0	10.0
Xiangjiao No.11	13.7±0.5	80.2±3.3	82.5±4.1	79.5±2.4	70.2±2.3	62.5±2.9
Ganjiao No.1	14.8±0.4	60.0±2.2	67.5±2.7	62.0±2.5	54.5±1.8	48.2±1.6
Atlantic	71.2±1.3	62.5±1.4	68.2±2.9	65.0±3.0	57.2±2.3	50.5±2.0
Zhongjiao No.7	34.6±1.2	85.2±3.7	95.0±4.1	90.2±3.4	86.5±2.6	76.2±3.1
Zhudachanglajiao	3.1±0.1	62.5±2.8	70.0±2.1	60.2±2.3	55.2±2.1	48.5±2.0
Tianza No.7	51.4±0.9	85.5±3.4	87.5±3.4	83.0±3.4	79.5±2.6	70.2±2.9
Jinyanlajiao	20.1±0.7	57.2±2.2	60.0±2.5	53.5±2.2	47.2±1.1	40.2±1.7
Chaotianjiao	15.3±0.4	50.0±2.0	52.5±2.1	47.5±2.1	42.5±1.8	34.5±1.2
Average	31.6	65.6	68.2	60.3	62.4	61.8

Effect of BA, IAA and GA₃ on Shoot Elongation

The influences of BA, IAA and GA₃ on the shoot elongation were studied by different combinations of the three plant hormones (Table 4, Figure 3). In the present experiment, some leaves without stems and rosette cluster shoot-bud which could not further develop to plantlet were discovered. The result was that all combinations of hormones have some effects on shoots elongation, but they were different. If only 0.5 mg L⁻¹ IAA or 1.0 mg L⁻¹ IAA was added to the medium containing 3.0 mg L⁻¹ BA, there is no considerable effect on the percentage of shoot elongation. After added with 1.0 mg L⁻¹ or 2.0 mg L⁻¹ GA₃, shoot elongation was greatly promoted with the frequency of 50.5 % and 53.1% on average, but there was no obvious difference between them, so 1.0 mg L⁻¹ GA₃ was used in the followed experiments. The highest frequency of shoot elongation was 72.5 % in Tianza No.7 after added with GA₃, increased about 2.5 times. Other cultivars varied, but the trend was the same as Tianza No.7. The percentage of elongated shoot increased a lot with IAA and GA₃ in the medium. Table 4 showed that, in general, the optimal medium for shoot elongation was MS medium+6.0 g L⁻¹ agar+30.0 g L⁻¹ sucrose +3.0 mg L⁻¹ BA+1.0 mg L⁻¹ IAA+1.0 mg L⁻¹ GA₃.

Table 4

Effect of various media on explants shoot elongation of different pepper varieties (%)

Cultivars	Media					
	A	B	C	D	E	F
Xiangjiao No.11	13.8±0.7	16.5±0.6	28.0±1.3	26.0±0.7	37.5±1.4	42.5±1.6
Ganjiao No.1	34.4±1.1	24.4±0.8	32.0±1.1	25.5±0.8	36.7±1.1	52.5±1.9
Atlantic	22.4±0.9	25.6±1.2	62.5±3.1	63.3±2.7	66.5±3.3	68.2±1.8
Zhongjiao No.7	23.5±0.8	28.5±1.0	55.0±2.6	62.7±2.5	67.2±3.0	65.0±1.2
Zhudachanglajiao	32.5±1.3	34.8±1.1	56.5±2.3	60.0±2.9	65.0±2.8	70.0±3.1
Tianza No.7	23.6±0.6	29.4±1.2	72.5±3.1	64.3±3.2	67.5±2.1	67.5±3.3
Jinyanlajiao	34.6±0.9	19.5±0.7	42.5±2.0	55.0±1.9	47.5±1.6	60.0±2.7
Chaotianjiao	13.5±0.4	27.5±1.0	55.0±2.1	60.3±2.4	45.0±1.2	67.5±3.1
Average	24.8	25.8	50.5	52.1	54.1	61.6

Note: A: 0.5 mg L⁻¹ IAA; B: 1.0 mg L⁻¹ IAA; C: 1.0 mg L⁻¹ GA₃; D: 2.0 mg L⁻¹ GA₃; E: 0.5 mg L⁻¹ IAA+1.0 mg L⁻¹ GA₃; F: 1.0 mg L⁻¹ IAA+1.0 mg L⁻¹ GA₃. ABCDEF have the same other ingredients: MS medium+6.0 g L⁻¹ agar+30.0 g L⁻¹ sucrose+3.0 mg L⁻¹ BA

Rooting of Elongated Shoots and Transplanting into Soil

When plantlets grown to 2 cm or so at length, they were cut at the ground of plantlets and transferred into the rooting medium. Two kinds of rooting medium were used in which one is MS medium and another is MS medium supplemented with 0.5 mg L⁻¹ IBA. Elongated shoots cultured in rooting medium for 2-3 weeks. Rooting in the medium containing IBA is faster than that in the medium without phytohormones.

In general, elongated shoots cultured in medium containing IBA for 15 d, or in MS medium for over 20 d. Then rooted plantlets were acclimatized with open cap before they were transferred into soil. Subsequently, plantlets were grown into maturity with a survival percentage of 90-95 %. They developed into normal plants and bearing normal fruits (Figure 4-5).

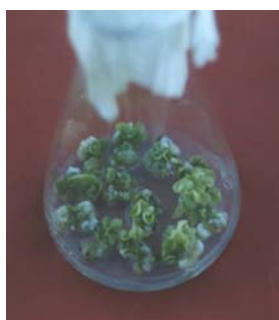


Figure 3. Explants on the elongated medium



Figure 4. Explants on the rooted medium



Figure 5. Plant before and after transplantation into soil

In recent years, there are many reports about pepper tissue culture with variable explants in which young organs and tissues are mostly used such as cotyledon, hypocotyls, stem apex, and young root even embryonic root (Ezura *et al.*, 1993). It is ideal that cotyledon

and hypocotyl are used as explants in which cotyledon is better.

Among the articles on *in vitro* plant regeneration of pepper, there is common trait using the plant growth regulators, and often BA is used as cytokinin and IAA as plant auxin (Fári, 1981; Phillips *et al.*, 1985; Agrawal *et al.*, 1989; Ochoa-Alejo *et al.*, 1990; Arrollo *et al.*, 1991; Valera-Montero *et al.*, 1992; Christopher *et al.*, 1996).

Based on above reason, the effects of concentrations and ratios of BA and IAA on shoot induction were studied. Eight pepper varieties all could differentiation on proper shoot induction medium, but they have difference in differentiation rates resulting from gene type, explant type, seedling stage and ingredients in the media (Dong Zhaolong, 2003).

The best formula for the medium is 5.0 mg L⁻¹ BA + 1.0 mg L⁻¹ IAA + 0.5 mg L⁻¹ nicotinic acid which make explants differentiate at high rates with good development. When concentration of nicotinic acid is above 0.5 mg L⁻¹ or below, differentiation frequency of explant is still comparatively high, but lower than 0.5 mg L⁻¹.

Hyde *et al.* (1996) observed that AgNO₃ influenced shoot induction of two cultivars. Explant could not regenerate in the medium containing cytokinin without AgNO₃. In order to confirm whether the action of AgNO₃ is affected by gene type or not, AgNO₃ was regarded as an induction-promotion factor added to the medium. The result shows that AgNO₃ could promote all cultivars explants differentiate rate used in the experiments without gene type specification, and other cultivars are under confirmation. Vain *et al.* (1989) considered that AgNO₃ was an ethylene inhibitor. Ethylene suppresses callus to differentiate and AgNO₃ eliminates the ethylene action, which, consequently, favors to explants differentiation.

Dabauza *et al.* (2001) combined GA₃ with BA, IAA and thidiazuron (TDZ), respectively. When TDZ was combined with GA₃, explant differentiated at highest frequencies. In the present experiments, the medium promoted shoot to elongate which contained BA, IAA and GA₃. That is to say GA₃ is an elongation-promotion factor, maybe it is because GA₃ has the function of promoting cell elongation.

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REZUMAT

EFECTUL REGULATORILOR EXOGENI DE CREȘTERE ASUPRA REGENERĂRII *IN VITRO* A EXPLANTELOR COTILEDONARE LA ARDEI IUTE

Au fost studiate plante regenerate obținute din explante prelevate din cotiledoane la opt soiuri de ardei iute (*Capsicum annuum* L.) și cultivate pe mediu de creștere bazal de tip Murashige - Skoog (MS) suplimentat cu diferiți regulatori de creștere (PGRs). Au fost urmărite patru pași-etape ale creșterii “*in vitro*” și “*in vivo*”, incluzând inducerea lăstarilor, elongarea (creșterea acestora), formarea rădăcinilor și transplantarea. Acidul nicotinic are un rol important asupra creșterii plantelor care poate induce diferențierea explantelor. AgNO₃ a sporit semnificativ frecvența formării lăstarilor la diferite soiuri de ardei iute. Acidul giberelic (GA₃) a constituit factorul cheie în elongarea lăstarilor. Creșterea lăstarilor pe mediul de cultură bazal MS cu acid indolil 3 butiric (IBA) a fost mai slabă decât pe același mediu bazal MS fără IBA. Plantulele au fost transplantate în sol și aclimatizate în seră, unde s-au dezvoltat normal. Studiile efectuate au permis stabilirea celei mai bune modalități pentru regenerarea *in vitro* a explantelor cotiledonare la ardei iute.