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杨国臣博士,1982年8月毕业于吉林农业大学蔬菜专业,分别于1989年和1993年在美国内布拉斯加大学获得硕士和博士学位。1994年至今就职于美国北卡洛莱纳A&T州立大学,副教授,生物技术学科带头人,生物技术委员会及本学科研究生委员会主席。荣获1999年美国农业部部长荣誉奖,2001年杰出青年科学家奖。1997年以来任美国农业部课题首席科学家。主要承担植物生物技术、植物组织培养、植物学、植物繁殖学等课程的教学任务。研究方向为植物组织培养,主要侧重于以下方面的研究:植物快繁,次生产物再生,利用组织培养快速筛选适合生物去污的植物材料以解决环保上的一些问题,利用组织培养和转基因的方法加强及发展生物能源的原材料。现有2项知识产权的保护,在美国以及其它国际有关学术会议上发表演讲百余次。

In Vitro Callus Induction and Shoot Initiation of American Chestnut (*Castanea dentata*)*

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Abstract: *In vitro* callus induction and shoot initiation of the American chestnut (*Castanea dentata*) were investigated in response to different plant growth regulators (auxins and cytokinins) in the culture medium. Micropropagated shoots were used as explant materials. Auxins included indolebutyric acid (IBA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Cytokinins included kinetin, N-(2-chloro-4-pyridyl)-N′- phenylurea (CPPU), thidiazuron (TDZ), and zeatin. Woody plant medium (WPM) salts containing 3% sucrose and 0.7% agar were used as the basic culture medium. Differences in the number of shoots (primodia), morphology of micropagated shoots, and amount of callus were observed between the plant growth regulator treatments and concentrations. Explants cultured in media containing cytokinins produced more shoots (primodia) with TDZ producing the most, followed by CPPU, kinetin and zeatin. Explants cultured in media containing auxins enhanced callus induction with 2,4,5-T achieving the most, followed by 2,4-D, IBA and NAA.

Key words: chestnut; auxin; cytokinin; tissue culture
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美洲板栗愈伤组织诱导和植株再生的研究

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摘　要：在培养基中添加不同植物生长调节剂（生长素和细胞机动素），对美洲板栗愈伤组织的诱导以及植株再生进行了研究。将试管繁殖的小植株切为长0.5 cm的小段作为外植体材料，以木本植物培养基加上3%的蔗糖和0.7%的琼脂作为基本培养基，再加上不同浓度的生长素或细胞机动素作为试验处理。生长素包括IBA，NAA，2，4-D和2，4，5-T；细胞机动素包括kinetin，CPPU，thidiazuron（TDZ）和zeatin。结果表明：外植体的植株再生和愈伤组织诱导百分率以及再生植株的形态发生以及愈伤组织的诱导量在不同的植物生长调节剂处理间和同一植物生长调节剂不同浓度的处理间都存在着显著的差异。促使外植体植株再生百分率最高的细胞机动素为TDZ，其次为CPPU，kinitin和zeatin；促使外植体愈伤组织诱导百分率最好的生长素为2，4，5-T，其次为2，4-D，IBA和NAA.

关键词：板栗；生长素；细胞机动素；组织培养
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The American chestnut [(*Castanea dentata* (Marsh.) Borkh], a member of the beech family (Fagaceae), was once the most agro-economically important forest hardwood timber species in the eastern United States[1]. But due to the fungal pathogen *Cryphonectria parasitica* (Murrill) Barr in the early 20th century it was virtually eliminated[2]. As a tree, the American chestnut was a valued resource. It provided lumber for building and a plentiful supply of chestnuts with low fat content that were used in various foods. The American chestnut tree was also the primary source of tannin, a compound used to treat and cure leather[3-4]. Furthermore, the leaves and the bark were used in herbal preparations. The chestnut tree was also a grand and graceful tree in maturity and was used throughout the east as a welcome landscaping.

In recent years through tissue culture and genetic transformation techniques considerable progress has been made towards the restoration of the American chestnut. Part of what is needed to be done is to take newly optimized plant cells and regenerate them into whole plants. This can be done using micropropagation system techniques to propagate them rapidly for large-scale field testing and eventually for commercial and restoration planting. Tissue culture procedures, such as micropropagation, have been demonstrated to be a valuable supplement to traditional breeding methods for enhancing disease resistance[5-6]. Micropropagation is a proven system of regeneration in forest biotechnology. Several millions of identical true-to-type individuals can be produced saving time and space.

Micropropagation has been practiced using field explants of the *Castanea* species. However, very limiting rooting was reported when microshoots of juvenile origin were cultured on Murashige and Skoog medium with one-half nitrates with either 0.5 or 1.0 mg/L IBA for 1—3 weeks followed by transfer to an auxin-free medium[7-8]. It was also reported that callus formation of *Castanea dentata* only occurred from winter buds when the medium was supplemented with auxin and kinetin[5,9]. There is no reported work describing plant regeneration of *Castanea dentata* from callus cultures.

Although some success[5,7,10-14] has been achieved using micropropagation techniques on explants of the *Castanea* species, there are currently no efficient or reliable protocols with which successful reintroduction of the American chestnut can be assured. To address this need the objective of this study was to improve *in vitro* regeneration efficiency of the American chestnut to provide a reliable production protocol for genetic trans-

formation, by investigating the effects of plant growth regulators and their concentrations.

1 Materials and Methods

1.1 Explant materials

Stock chestnut cultures were initiated and maintained on WPM with 3% sucrose, 0.7% agar and 0.1 mg/L BA. Micropropagated shoots were cut to about 0.5 cm (1/4 inch) in length and used as explant materials for this research.

1.2 Basic culture medium and culture conditions

Lloyd and McCown's woody plant medium salts containing 3% sucrose were used as the basic culture medium[15]. The pH of all the media was adjusted to 5.8 using 0.1 mol/L KOH or 0.1 mol/L HCl before the addition of 0.7% agar and autoclaving at 121℃ (106 kPa) for 20 min.

Cultures were then incubated under 30—40 μmol/(s·m^2) and 16 h photoperiod provided by cool white fluorescent tubes at (23 ± 1)℃. Explant samples were cultured in individual glass culture vials containing 10 mL of each plant growth regulator or concentration treatment medium. All cultures were transferred to fresh media every four weeks.

1.3 Plant growth regulators for shoot initiation and callus induction

Four auxins (IBA, NAA, 2,4-D, and 2,4,5-T) at 0.5, 1.0, 1.5, 2.0 mg/L, and four cytokinins (CPPU, kinetin, TDZ and zeatin) at 0.1, 0.5, 1.0, 2.0 mg/L were added separately to the basic culture medium to study their effects on shoot initiation.

1.4 Experimental design and data analysis

A completely randomized design was used to assign the plant growth regulators at their various concentration levels. There were 36 culture vial replications per plant growth regulator concentration treatment. Each culture vial contained one explant. Data on percent of explant regenerating shoots and callus induction were analyzed using SAS to determine the influence of plant growth regulators and concentration treatments. The treatment differences were separated using Least Significant Difference (*LSD*) at $\alpha = 0.05$ level. Morphological characteristics were also recorded and compared among the treatments.

2 Results and Discussion

2.1 Effect of growth regulators and concentrations on shoot initiation

Cytokinins and their concentration levels significantly influenced *in vitro* shoot growth and development. Among the four cytokinins tested, zeatin was associated with the lowest shoot multiplication, while TDZ and CPPU had the highest (Table 1). In terms of concentration levels, both TDZ and CPPU at 0.1 and 1.5 mg/L produced the best shoot multiplication. This finding is supported by other researchers with other plant species who recorded an increase in shoot multiplication rate by increasing the level of cytokinin up to a certain value depending on the micropropagated plant and culture conditions[16-17]. The basis for the increase is likely due to the cytokinin stimulatory effect on cell division and enlargement. Differences in the number of shoots (primodia) and morphology of micropropagated shoots were observed between the cytokinin treatments and concentrations within each cytokinin. Explants cultured in media containing cytokinins produced numerous shoots and/or clusters of primodia. However, there were variations in the color of the shoots produced from cytokinins media. Explants from the CPPU or TDZ treatments swelled and produced numerous shoots and/or cluster of primodia that were purple or red-purple in color, while explants receiving the zeatin and kinetin treatments produced considerably less axillary shoot with regular green color (data not shown). Lower concentrations of TDZ resulted on many short adventitious shoots (primodia), whereas the higher concentration of TDZ resulted in low number of shoot primodia.

Table 1. Shoot initiation of American chestnut explants cultured on WPM plus cytokinins at different concentrations for 8 weeks

Concentration/(mg·L^{-1})	Explants regenerating shoots/%			
	TDZ	Zeatin	Kinetin	CPPU
0.1	84	29	47	74
0.5	75	35	41	68
1.5	88	28	54	76
2.0	55	35	29	65
$LSD^{*}_{0.05}$	14	7	13	10

* *LSD* values are for comparison within each cytokinin only

Measurable shoot proliferation was also observed in explants in media containing auxins. The results clearly indicate that medium containing auxins with low (0.5 mg/L) IBA was greatly favored for shoot initiation (Table 2). As the IBA concentrations increased the number of the shoot production decreased. The axillary shoot proliferation was the lowest with the culture medium containing NAA or 2,4,5-T. It appears that shoot production had a tendency to increase at low concentrations but decreased considerably at the highest concentration (2.0 mg/L). These results might be due to the combination effects of BA and IBA because the stock cultures were maintained in WPM containing 0.1 mg/L BA. Variations in the shoot morphological characteristics of these micropropagated shoots were observed between the plant growth regulator treatments and concentrations (data not shown). Shorter shoots and leaves developed at higher concentration, which was almost consistent with the result obtained for shoot production. A similar pattern was observed in the color of the shoot; explants cultured on the medium with IBA or 2,4-D produced green and healthy shoots, whereas those shoots developed in NAA or 2,4,5-T had a greenish-yellow color.

Table 2. Shoot initiation of American chestnut explants cultured on WPM plus auxins at different concentrations for 8 weeks

Concentration/(mg·L^{-1})	Explants regenerating shoots/%			
	IBA	NAA	2,4-D	2,4,5-T
0.5	72	15	67	3
1.0	45	7	42	13
1.5	63	3	31	6
2.0	14	3	10	3
$LSD^{*}_{0.05}$	13	5	11	5

* *LSD* values are for comparison within each auxin only.

We also noticed shoot apex necrosis in our chestnut cultures, which could seriously hamper the progress of tissue culture research. Previous studies have also noted severe loss of cultures due to apex necrosis for chestnut[7,12,18-19]. Shoot apex necrosis has been considered a physiological disorder for American chestnut and European chestnut[19-20]. Exudation of phenolics had been reported to cause necrosis of shoot tips in *in vitro* culture of many tree species. Along with the hormonal hypothesis, deficiency of calcium or boron had been suggested as the most likely cause of tip necrosis[21-22].

2.2 Effect of plant growth regulators on *C. dentata* callus initiation

Four different auxins and cytokinins and concentrations were investigated on callus induction. The results showed that the medium containing 2,4,5-T at 0.5 mg/L was greatly favored for callus initiation (Table 3). As the 2,4,5-T concentration increased, the percentage of explants producing callus decreased. The callus induction was the lowest with the culture medium containing NAA, although there were reports that NAA induced good-quality callus in a number of

medicinal plants[23]. Our results indicate that callus induction decreased at higher concentrations of auxins, but increased considerably at the lowest concentration (0.5 mg/L). During the experiment differences were observed on percentage explants with callus. Between 28% and 84% of explants of *C. dentata* produced callus when the explants were cultured on media containing different auxins with 2,4,5-T and 2,4-D the highest and NAA the lowest.

Table 3. Callus induction of American chestnut explants cultured on WPM plus auxins at different concentrations for 8 weeks

Concentration/(mg·L^{-1})	Explants producing callus/%			
	IBA	NAA	2,4-D	2,4,5-T
0.5	53	52	68	84
1.0	61	59	74	77
1.5	47	28	72	81
2.0	51	41	67	58
$LSD^{*}_{0.05}$	8	12	6	18

* *LSD* values are for comparison within each auxin only

Auxins in the culture media also influenced callus' color and the other morphological characteristics (data not shown). Callus produced from explants cultured in medium containing 2,4,5-T was compact and greenish-white than callus produced on the media containing IBA, NAA or 2,4-D. The callus appeared less thick and yellowish-white from explants cultured on IBA, NAA or 2,4-D. However, the callus induced from cultured media did not regenerate into shoots. Extended incubation over a extended period of time on medium containing 2,4,5-T resulted in the callus turning brown.

It is normally believed that cytokinins induced shoot initiation. However, we noticed from our research that callus induction was achieved when explant materials cultured in media containing different cytokinins at various concentrations (Table 4). The percentage of explants producing callus was quite significant between as high as 41% and as low as 3% among the cytokinins and concentrations treatments. The results indicated that callus proliferation was of a lower percentage when CPPU was used instead of kinetin. Kinetin in the medium appeared to have effect on callus induction of chestnut. The increases in shoot number in the media containing cytokinins were associated with a progressive decrease in callus production.

Table 4. Callus induction of American chestnut explants cultured on WPM plus cytokinins at different concentrations for 8 weeks

Concentration/(mg·L^{-1})	Explants producing callus/%			
	TDZ	Zeatin	Kinetin	CPPU
0.1	28	29	31	12
0.5	22	9	41	15
1.5	41	13	36	3
2.0	27	12	41	8
$LSD^{*}_{0.05}$	11	14	7	6

* *LSD* values are for comparison within each cytokinin only

3 Conclusions

Our results indicate that CPPU and TDZ at low concentrations significantly enhanced *in vitro* shoot initiation of the American chestnut. Some auxins in the culture media also increased percentage of explant producing shoots. This result might be due to the combination effects of BA and IBA because the stock cultures were maintained in WPM containing a low concentration of BA. The appropriate combination of an auxin

and a cytokinin could further improve the efficiency of in vitro shoot proliferation of American chestnut. Contrary to conventional belief, we observed that cytokins in the culture medium also had an effect on callus induction of American chestnut. While the results of this study demonstrated that the effectiveness of selecting different plant growth regulators, further research is needed to confirm our observations and to further investigate the interaction of auxins and cytokinins for improving *in vitro* regeneration efficiency of American chestnut.

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