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A MICROPROPAGATION SYSTEM FOR CLONING OF HEMP (*CANNABIS SATIVA* L.) BY SHOOT TIP CULTURE

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Abstract

This study describes the standardization of an efficient *in vitro* propagation and hardening procedure for obtaining plantlets from shoot tips of Hemp (*Cannabis sativa* L.). Hemp seedlings were germinated on half-strength 1/2 MS medium supplemented with 10 g·L^{-1}sucrose, 5.5 g·L^{-1}agar at a pH of 6.8 under light for 16 h per day. MS medium containing 0.2 mg·L^{-1}TDZ, 0.1 mg·L^{-1}NAA supported the maximal auxiliary bud multiplication rate of 3.22 per shoot tip. The proliferated buds were successfully rooted on MS medium supplemented with 0.1 mg·L^{-1}IBA and 0.05 mg·L^{-1}NAA resulting in 85% of the plantlets rooting. The procedure requires a 54 days cycle for the *In vitro* clonal propagation (14 days for shoot multiplication and 40 days for root induction) which includes 35-42 days for acclimatized plantlet production.

Introduction

Hemp (*Cannabis sativa* L.) belongs to the Cannabidaceae family. It is an annual herb that has been cultivated for the value of its fiber, and more recently for paper manufacturing, oil extraction and medicinal or drug preparation (Ranalli *et al*., 1999). In the 19th century, other fibre-producing plants such as cotton, jute and sisal became more competitive and caused a decline in hemp production (Rode *et al*., 2005). However, recently the interest in hemp has been renewed due to its adaptability to a wide variety of agro-ecological conditions, its high yields compared with many other crops and the production of medicinal components such as tetrahydrocannabinol (THC) (Struik *et al*., 2000).

There are few research reports about the tissue culture of hemp. Most of these studies were aimed at developing a cell culture system to obtain secondary metabolites, particularly the THC class of cannabinoids that are distinctive to the genus of *Cannabis* (Turner *et al*., 1980). Callus induction and a protocol for *Agrobacterium* mediated transformation of hemp had been established (Hartsel *et al*., 1983; Braemer & Paris, 1987; Feeney & Punja, 2003), but no micro-propagation system had been reported until now. In order to provide a basis for advancing the fundamental biology of hemp and abundant raw materials for industrial production, the objective of this study was to identify the condition for auxiliary bud induction and rooting of hemp to promote regeneration of plantlets from shoot tips.

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Materials and Methods

Plant material: Hemp (*Cannbis satival L*) varieties Changtu from Liaoning province, China, were selected for this study. Hemp seeds were first washed for 20 min with 0.1% antiseptic liquid detergent APSA80 (Amway Corporation, Ada, Michigan, USA). The seeds were made free from the detergent foam by thoroughly washing under running tap water for 60min., in beaker. The washed seeds were surface sterilized by briefly dipping in 75% (v/v) ethanol for 30 s followed by four washes with sterile distilled water and subsequent sterilization for 10-15 min., with 0.1% Mercuric chloride. The treated seeds were finally rinsed 8 times with sterile distilled water to remove traces of sterilizing agents. The sterilized seeds were germinated on 1/2 MS medium with 10 $g \cdot L^{-1}$sucrose, 5.5 $g \cdot L^{-1}$agar, pH 6.8, under light for 16 h per day.

Auxiliary bud and root induction: Two sets of experiments were conducted for auxiliary bud induction. In both case, shoot tips were harvested from 20-day-old sterile plantlets. The shoot tips were grown *In vitro* on MS medium (Murashige & Skoog, 1962) supplemented with BA (1.0, 2.0, 5.0 $mg \cdot L^{-1}$), KT(1.0, 2.0, 5.0 $mg \cdot L^{-1}$) or TDZ(0.1, 0.2, 0.5 $mg \cdot L^{-1}$) respectively in Experiment I and 0.05, 0.1, 0.5 $mg \cdot L^{-1}$NAA in Experiment II. Sterile plantlets produced from the shoot tips were then rooted introduction to free-of basic nutrition medium supplemented with 0.1, 0.5 $mg \cdot L^{-1}$IBA respectively or in combination with 0.05, 0.25 $mg \cdot L^{-1}$ of NAA or IAA (Table 1). In order to choose the optimal medium for root development, the plantlets were introduced to 1/2MS, MS, B5 (Gamborg *et al*., 1968) or NN (Nitsch & Nitsch, 1969) with the optimal level of plant growth regulators (Table 1).

Culture conditions: All the media for auxiliary bud induction and plantlet root production contained 30 $g \cdot L^{-1}$ sucrose as carbon source, 6.8 $g \cdot L^{-1}$agar. The pH of the medium was adjusted to 5.8 before sterilizing in an autoclave at a temperature of 121^{o}C and at the pressure of 151 bs psi for 15 min. The cultures were kept at 25±1^{o}C with 16 h light cycle of a light intensity of 2500 lux in every 24 h. Each treatment comprising of 20 explants pieces was repeated twice.

Hardening and transplantation: The *In vitro* raised plantlets were deflasked, when the length of the roots reached 0.5-1.0 cm. One day after deflasking, 10 ml of sterile water was poured into the bottle with cultured plantlets of hemp. The plantlets were cultured for a further week in the bottle after which the hemp plantlets were thoroughly washed in running tap water so as to remove any adhering medium from the surface of the plantlets followed by washings with 0.2% (w/v) Bavistin1 and tap water. The washed plantlets were transferred to root trainers consisting of 20 cells, each of 200 cm^3, filled with perlite and 10 ml water and cultured for an extra 2-3 weeks. Finally, the plantlets were transferred to plastic pot, filled with vermiculite and plant ash and grown in shade-house. After an acclimatization period of 2 weeks, the plantlets were able to be transplanted in field.

Statistical analysis: The successful production of viable plantlets was evaluated at 40 days, and the data was analyzed by SPSS software, version 10.0 (Supplier, location) by analysis of variance (ANOVA). The mean differences were tested using 'F' test at $p<0.05$ level.

Result and Discussion

Auxiliary bud induction: Aseptic shoot tips were introduced to MS medium supplemented with different types of cytokinin for auxiliary bud induction (Fig. 1A). Table 2 shows the effect of different concentrations of BA, KT and TDZ on shooting response in the shoot tips of hemp. Cytokinin stimulated shoot formation and stem enlargement for each explant in these nine treatments. Among the three cytokinins tested, TDZ (0.2mg·L^{-1}) was found to provide the best bud induction, inducing an average of 3.22 buds with the thickest stem (Fig. 1B). Furthermore, the type of cytokinin in the medium also affected plantlet morphology, with the plantlets grown in TDZ-containing medium being more compact and vigorous. The suitability of TDZ for *In vitro* auxiliary shoot propagation has been well established in many woody plants tissue culture (Carl & John, 1993), and also determinated in many herbage plants (Donna & John, 2004; Chitra & Padmaja, 2005; Peddaboina *et al*., 2006).

Table 3 shows the effect of different NAA concentrations on the growth of auxiliary buds, where the supply of 0.1 mg·L-1 NAA concentration was optimal for auxiliary bud induction.

Table 1. The effect of different hormones on rooting.

Treatment	IBA (mg·L^{-1})	NAA (mg·L^{-1})	IAA (mg·L^{-1})	Root number	Root length	Stem wide
1	0.1	–	–	0.65	+	++
2	0.1	0.05	–	2.45	++	+++
3	0.1	0.25	–	1.45	+++	+++
4	0.1	–	0.05	0.80	++	+++
5	0.1	–	0.25	1.50	++	+++
6	0.5	–	–	0.85	++	+++
7	0.5	0.05	–	0.50	+	+++
8	0.5	0.25	–	0.85	++	+++
9	0.5	–	0.05	1.05	++	++
10	0.5	–	0.25	1.95	++	++

+ Short or thin, ++ Longer or thicker, +++ Longest or thickest

Table 2. Effect of BA, KT and TDZ treatments on plantlet formation.

Hormone	Concentration (mg·L^{-1})	Auxiliary bud number	Stem wide
BA	1.0	2.43bc	+ +
BA	2.0	1.65bc	+ + +
BA	5.0	1.57c	+ +
KT	1.0	1.83bc	+ +
KT	2.0	2.00bc	+ +
KT	5.0	1.74bc	+
TDZ	0.1	2.87ab	+ + +
TDZ	0.2	3.22a	+ + +
TDZ	0.5	2.35bc	+ +

Auxiliary bud number followed by the same letters are not significantly different at $p \leq 0.05$ by F test.
+ Thin, ++ Thicker, +++ Thickest

Fig. 1A The 20-day-old sterile plantlet of apical buds inoculated on MS medium containing 0.2 mg/L TDZ + 0.1 mg/L NAA (2.5 X).

Fig. 1B Auxiliary buds plantlets. Induction of shoot multiplication after 14 days of inoculation on MS medium containing 0.2 mg/L TDZ + 0.1 mg/L NAA (2.0 X).

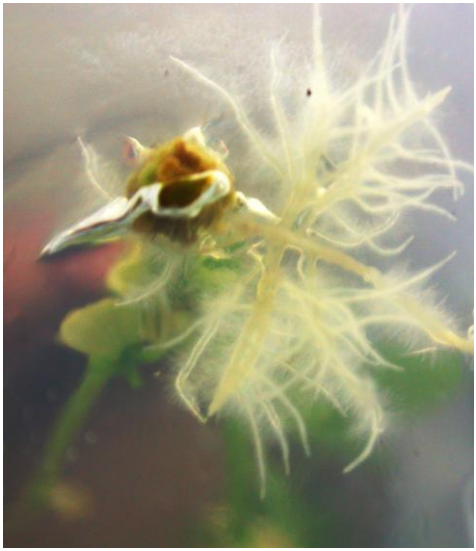

Fig. 1C Auxiliary buds in rooting. *In vitro* root formation after 10 days of inoculation on MS medium containing 0.1 mg/L IBA + 0.05 mg/L NAA (2.5 X).

Fig. 1D Integrated plantlets. *In vitro* root formation after 30 days of inoculation on MS medium containing 0.1 mg/L IBA + 0.05 mg/L NAA (1.0 X).

Root establishment: When the regenerated auxiliary buds were separated and transferred to the rooting medium with different hormones, rhizogenesis occurred followed by enlargement of the stem during 40 days culture. The effects of environmental and medium factors on rooting were investigated by evaluating the rooting percentage, root length, and number of roots per plantlet. The combination and concentration of auxin forms significantly influences the number of roots formed (Table 1). The best rooting and elongation occurred with treatment 2 that contained 0.1 $mg \cdot L^{-1}$IBA and 0.05 $mg \cdot L^{-1}$NAA (Fig. 1C). The combination of these two auxins for increasing rooting has previously been

observed (Amin *et al*., 1987; Kornova, 1995). ANOVA evaluation (Table 4), showed that the F value was higher than the 0.05 $mg \cdot L^{-1}$ level of these auxins, demonstrating that different treatments supplemented with different combinations and concentrations of hormones have distinct effects on plantlet rooting performance (Table 4).

Four intermediate formulations, 1/2MS, MS, B5 and NN media, were tested for growth media for rooting culture. Both the basic media of 1/2MS and MS were found to have a superior root proliferation rate of 75% and 85%, while the B5 and NN basic media did not differ in root length and root number performance (Table 5). The induction of root numbers was maximal in those plantlets cultured on MS (2.45 roots/plantlet), followed by those on 1/2MS (2.05 roots/plantlet), B5 (0.85 roots/plantlet) and NN medium (0.60 roots/plantlet) (Fig. 1D).

Hardening and transplantation: The well grown *In vitro* plantlets, after treatment with 0.2% (w/v) Bavistin1, were hardened by the following two-step procedure: (i) controlled conditions of culture room for 3-4 weeks and (ii) semi controlled conditions in the shade house for 2 weeks allowed 95% of plantlets to acclimatize, after which 99% of these plantlets were surviving for 3 months after transfer to the field.

Table 3. The effect of different NAA concentrations on the growth of auxiliary buds.

Hormone	Concentration ($mg \cdot L^{-1}$)	Auxiliary bud number
NAA	0.05	1.2 b
NAA	0.1	2.4 a
NAA	0.5	1.1 b

Auxiliary bud number followed by the same letters are not significantly different at $p \leq 0.05$.

Table 4. The analysis of variance of different hormones effect on rooting.

Variation source	DF	SS	MS	F Value	F0.05 Level	F0.01 Level
Treatment	9	69.95	7.77	2.39	1.97	2.59
Error	190	618.65	3.26			
Collected total	199	688.6				

Table 5. The effect of different media on rooting efficiency.

Basic medium	Root number	Root %	Root length	Stem wide
1/2MS	2.05 a	75.0	+++	+
MS	2.45 a	85.0	++	+++
B5	0.85 b	20.0	+	++
Nitsch	0.60 b	25.0	++	++

Root numbers followed by the same letters are not significantly different at $p \leq 0.05$ by F test.
+ Short or thin, ++ Longer or thicker, +++ Longest or thickest

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