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An Efficient *In vitro* Propagation of *Zanthoxylum piperitum* DC.

Sung Jin Hwang*† and Baik Hwang**

*Department of Food and Biotechnology, Dongshin University, Naju 520-714, Korea.
**Department of Biology, Chonnam National University, Kwangju 500-757, Korea.

ABSTRACT : A protocol is described for rapid multiplication of *Zanthoxylum piperitum* DC. (Rutaceae), an important aromatic and medicinal plant, through shoot-tip explant cultures. Murashige and Skoog (MS) medium supplemented with various concentrations of N-6-benzyladenine (BA), N-6-benzylaminopurine (BAP) and thidiazuron (TDZ), in single or in combination with α-naphthaleneacetic acid (NAA), was used to determine the rate of shoot proliferation. N-6-benzyladenine (BA) used at 0.5 ㎎/ℓ was the most effective in initiating multiple shoot proliferation at the rate of 23 microshoots per shoot-tip explants after 40 days of culture. Shoot multiplication increased 1.2-fold in each successive subculture. Induction of rooting (98%) was achieved by transferring the shoots to the same basal medium containing 2 ㎎/ℓ indole-3-butyric acid (IBA). Plantlets went through a hardening phase in a controlled growth chamber, prior to *in vivo* transfer. These results represented that possible application for the mass production of plantlets through *in vitro* culture system of *Zanthoxylum piperitum* DC.

Key words : *Zanthoxylum piperitum* DC., shoot-tip culture, *in vitro* propagation

INTRODUCTION

Zanthoxylum piperitum DC. (Rutaceae) distributed in Korea, Japan, and China is a strong aromatic plant used as traditional spices and a medicinal plant against cold, hypotension, neuralgia, paralysis and toothache (Go & Han, 1996). Some of the biological activities detected in this species may possible include anticancer effect (Kimura *et al.*, 1996). The GC-MS results of *Z. piperitum* revealed the presence of over 100 volatile components. Major components were 1,8-cineol, limonene, geranyl acetate, and myrcene in the fruit peel and citronellal, 1,8-cineol, and citronellol in leaf (Kim *et al.*, 1989). Since the concentrations of volatiles and other compounds are different for each plant, the main goal of breeding is to select highly productive individuals and to propagate them vegetatively in order to maintain their valuable characteristics. Conventional propagation through seeds and cuttings of stem and root is too slow to provide the answer to the demand for this valuable plant in time. Propagation through seeds is an inadequate solution due to low viability, a low germination rate, and delayed rooting of the seedlings. Clonal propagation of plant germplasm through tissue culture for rapid production of medicinal plants is an important prerequisite for *in vitro* conservation (Lynch, 1999). Successful micropropagation of tree species is a relatively recent phenomenon (Mott, 1981; Thorpe, 1990; Bajaj, 1997). Several woody species such as poplas, eucalyptus, and wild cherry have been supplied in a massive scale (Gavinlertvatana *et al.*, 1987; Thorpe, 1990; Bajaj, 1997), while for others such as loblolly pine, birch, and Shorea, the protocols are being standardized for mass multiplication (Haissig *et al.*, 1987). So far, no information is available on micropropagation of *Z. piperitum*. In this paper we describe the development of an *in vitro* propagation system for *Z. piperitum*.

† Corresponding author : (Phone) +82-61-330-3225 (E-mail) jinsci@lycos.co.kr
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MATERIALS AND METHODS

Plant materials

Seeds of *Z. piperitum* DC. were collected from trees growing in the forests at Kwang-yang, south Korea. They were classified by Department of Biology, Chonnam National University.

Sterilization and germination of seeds

The seeds were surface-sterilized with an 70% (v/v) ethanol for 5 min., and aqueous solution of 4% (v/v) sodium hypochlorite plus 1~2 drops of Tween 20 for 10 min. and then rinsed five times for 10 min. in sterile distilled water. After sterilization, seeds were cultured on MS (Murashige & Skoog, 1962) basal medium supplemented with 2% (w/v) sucrose and 0.3% (w/v) Phytagel (Sigma Co., USA). In all cases, the pH was adjusted to 5.7 with 1 N NaOH before autoclaving at 121℃ and 105 kPa for 20 min. The cultures were incubated at 26℃ and 16h photoperiod under cool white fluorescent light (40 μmol $m^{-2}s^{-1}$). These culture conditions were used in all the experiments mentioned below unless otherwise stated.

Shoot multiplication

After 3 months in culture, new shoots from explant cultures were excised approximately 1~1.5 ㎝ long and transferred into shoot induction medium. The medium used was MS, B_5 (Gamborg *et al.*, 1968) or WPM (Lloyd & McCown, 1981) containing 30 g/ℓ sucrose and 3 g/ℓ Phytagel and supplemented with BA, BAP, or TDZ at the concentrations of 0.5, 1.0, or 2.0 ㎎/ℓ in combination with 0~0.5 ㎎/ℓ NAA (Table 1). Explants were oriented in the vertical position in the culture media and replicated 3 times. The percentage of explants producing shoots and the average number of shoots produced per explant were recorded after 6 weeks.

Table 1. Effect of various culture media on *in vitro* shoot proliferation of *Z. piperitum*.

Media	No. of microshoot/explant	Explant initiating shoot (%)
MS	23.4 ± 1.5†	98.2
WPM	15.7 ± 2.1	78.3
B_5	3.4 ± 1.3	42.3

†Data (mean±SD) scored after 6 weeks for 20~25 explants per treatment.

Rooting and acclimatization

In order to induce the rooting, shoots obtained with the concentration of plant growth regulators giving rise to maximal shoot production were excised and cultured in hormone-free MS basal medium or the MS medium supplemented with IBA or NAA at the concentrations of 0.5, 1.0, 2.0, or 3.0 ㎎/ℓ, respectively. After 6 weeks of culture, the rooting percentage, the number of roots and the maximum length of roots were evaluated.

RESULTS AND DISCUSSION

Twenty percent of *Z. piperitum* DC. seeds germinated within 2 weeks of culture on MS basal medium. Since Morel (1960) succeeded in the rapid propagation of orchids by shoot meristem culture, techniques for *in vitro* propagation of many plant species have become routine. The propagation of shoots was regulated by the culture condition. The important factors were reported kinds of the plant growth regulators added to the culture medium, the concentration of carbon source, the strength of macronutrients, and the age of explants. Initially the shoot-tip explants were cultured on various basal media, viz. MS, B_5, or WPM. On all the media, the new shoots developed within 20~30 days of inoculation. Among the three basal media, MS proved to be the best, where 98% of explants formed new shoots with an average of 23.4 ±1.5 shoots per explant (Table 1) and an average shoot length of 1~1.5 ㎝ (data not shown). Therefore, MS medium was used for the shoot multiplication.

To optimize the level of sucrose on the shoot multiplication, MS medium was supplemented with different concentration (1~5%) of sucrose. In the absence of sucrose, shoot development was inhibited. The 2~3% of sucrose proved to be the most effective of concentration tested for *in vitro* shoots culture of *Z. piperitum* (Table 2).

Table 2. Effect of sucrose concentration on *in vitro* shoot proliferation of *Z. piperitum*.

Sucrose	No. of microshoot/explant	Explant initiating shoot (%)
1	14.3 ± 0.3	48.2
3	23.4 ± 1.5	98.2
5	11.3 ± 1.3	42.3

*Data (mean±SD) scored after 6 weeks for 20~25 explants per treatment.

Culture of excised new shoot from germinated shoot explants on MS, B_5, or WPM media supplemented with various concentration of BA, BAP, TDZ, single or in combination with NAA resulted in shoot formation; however, the number of shoot formed varied with the treatment. Among the cytokinins used, BA was observed to be more suitable than other cytokinins or combination with NAA for initiating shoot buds (Table 3).

Table 3. Effects of plant growth regulators on *in vitro* shoot proliferation of *Z. piperitum*.

PGRs (mg^{-1})				No. of microshoot /explant	Explant initiating shoot (%)
NAA	0.0	BA	0.5	23.4 ± 1.5†	98.2
			1.0	17.3 ± 1.9	87.6
			2.0	6.3 ± 0.7	31.2
NAA	0.5	BA	0.5	-	-
			1.0	2.1 ± 0.7	23.4
			2.0	8.3 ± 0.5	28.2
NAA	0.0	BAP	0.5	14.5 ± 1.5	61.0
			1.0	13.7 ± 1.7	58.4
			2.0	-	-
NAA	0.5	BAP	0.5	-	-
			1.0	-	-
			2.0	11.7 ± 1.6	43.4
NAA	0.0	TDZ	0.5	-	-
			1.0	7.2 ± 0.7	38.4
			2.0	5.0 ± 0.6	28.3
NAA	0.5	TDZ	0.5	-	-
			1.0	-	-
			2.0	12.2 ± 1.3	43.7

†Data (mean±SD) scored after 6 weeks for 20~25 explants per treatment.

The kinetin was not effective in inducing shoot bud differentiation (data not shown). BA alone significantly increased the number of shoots. The rate of multiplication was found to be maximal (23 shoot buds per shoot) on the medium supplemented with 0.5 ㎎/ℓ BA (Fig. 1). Shoot length, however, was not affected at any BA concentration. In general, the kind and balance of auxin and cytokinin are the one of important factors for shoots multiplication(Skoog & Miller,1957; Walker *et al.*, 1979). In this experiment, BA was the most effective component among the cytokinins for shoot induction. Proliferated shoots were subcultured for every 3~4 weeks, and new shootlets were harvested periodically. The production of shoots was further promoted by repeated subculturing of original explants on the fresh multiplication medium supplemented with 0.5 ㎎/ℓ BA after each harvest of the newly formed shoots. It was clearly indicated that induction of *in vitro* shoot multiplication in *Z. piperitum* can be accomplished relatively by using shoot tips isolated from aseptic seedling.

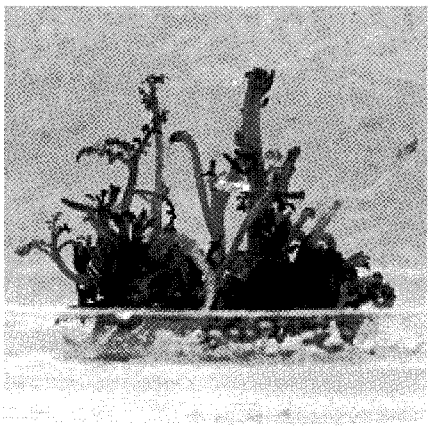

Fig. 1. Shoot multiplication in MS medium supplemented with 0.5 ㎎/ℓ BA.

Different plant species might vary in their requirement of auxin type for adventitious root formation. Elongated shoots in 2~2.5 ㎝ long were excised to test the effects of various auxin treatments on root induction. As shown in Table 4, IBA was more suitable for root induction than NAA (Fig. 2). Root length showed only very minor differences among the various auxin concentrations (data not shown). When cultured on

MS medium supplemented with 2 ㎎/ℓ IBA alone, each shoot developed an average of six roots. Rooting and transplantation of plantlets to the field is the most important but different task in micropropagation (Murashige, 1974). For efficient micropropagation system in a breeding program, a high frequency of successful rooting and establishment in the soil are necessary. The effectiveness of IBA in rooting has been reported for medicinal plants like *Hemidesmus indicus* (Sreekumar *et al.*, 2000), *Heracleum candicans* (Sharma & Yelen, 1995), and *Taxus mairei* (Chang *et al.*, 2001). The addition of NAA alone or IBA with NAA facilitated callus formation. According to Nickell (1982), the slow movement and degradation of IBA facilitates its localization near the site of application and thus its better function in inducing roots.

Table 4. Effect of auxins on rooting of *in vitro* formed shoots of *Z. piperitum.*

Auxins (mg^{-1})		No. of root/ shoot explant	Rooting shoot (%)
Control		1.2 ± 0.3†	23.4
IBA	1.0	2.9 ± 0.4	87.1
	2.0	6.2 ± 0.3	98.7
	3.0	1.2 ± 0.6	55.4
NAA	1.0	-	-
	2.0	0.7 ± 0.4	21.8
	3.0	-	-

†Data (mean±SD) scored after 6 weeks for 20~25 explants per treatment.

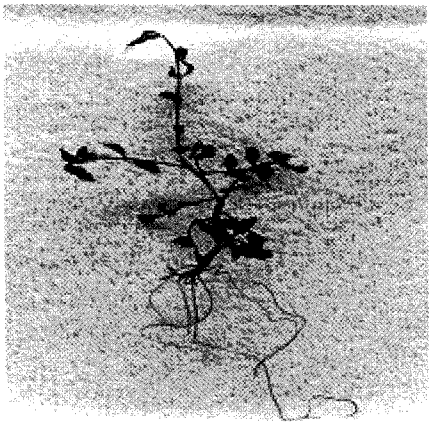

Fig. 2. Rooted shoot in MS medium supplemented with 2 ㎎/ℓ IBA.

The protocol described here for the micropropagation of *Z. piperitum* through microshoot multiplication facilitates the rapid propagation of this valuable medicinal plant. It will also be of use in conservation and genetic transformation studies aimed at improving the plant.

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