J. Plant Biotechnology(2004) Vol. 6(3). pp. 157~163

Journal of Plant Biotechnology

In Vitro Regeneration of *Lycium chinense* Miller and Detection of Silent Somaclones with RAPD Polymorphisms

In-Suk Ahn[1], Young Goo Park[2], Dong-Ill Shin[3], Ill-Whan Sul[4*]

[1]Department of Forestry, North Carolina University, USA; [2]Department of Forest, Kyungpook National University; [3]Department of plant genetic engineering, Daegu Catholic University; [4]Department of Biotechnology, Daegu University of Foreign Studies

Abstract

An efficient system for the regeneration of adventitious shoots from *in vitro* cultured leaf sections of *Lycium chinense* Miller was developed and silent somaclones from the regenerants detected with RAPD method. Among the eight media tested (B5, SH, N&N, 1/2MS, MS, 3/2MS, GD and WPM), and four cytokinins (BA, kinetin, 2ip and zeatin) with different concentrations (1, 5, 10, 20, 30 and 40 μM), 1/2 MS medium supplemented with 20 and 30 μM zeatin showed the best regeneration frequency (100% and 93.7%) and higher average number of shoots (9.0 and 9.4). All regenerants easily elongated after subculturing on 1/4MS without growth stimulants and produced spontaneous adventitious roots from their basal parts. With phenotypically normal 40 regenerants, RAPD analysis with 15 different random primers was performed to examine the cryptic somaclonal variants. No substantial differences in banding patterns were found in the amplified polymorphic DNAs implying no DNA changes during dedifferentiation into adventitious shoots. However, one (OPF-4) of the 15 primers detected silent somaclonal variation in one regenerant in which two different polymorphic bands did not appear when compared with the rest regenerants. The results indicate that regenerantion via intervening callus phase can be used to establish true-to-type planting stocks for homogeneous population.

Key words: *Lycisum chinense*, regeneration, RAPD, somaclones

* Corresponding author, E-mail: iwsul@dufs.ac.kr
Received Mar. 31, 2003; Accepted Aug. 25, 2004

Introduction

Lycium chinense Miller, a member of Solanaceae, is commercially important for mainly medicinal purposes and daily beverage in Asia. Dried fruits, seeds, stems and roots contain valuable medicinal compounds such as luteole, niacin, vitamins, minerals, betain and other amino acids. They can tonify spleen and kidney, and are used for curing blood insufficiency, dizziness, diabet, hypertension and antihepatotoxic agent (Chin et al. 2003).

Clonal propagation and genetic improvement in *L. chinense* have been achieved mostly through the traditional breeding methods. However, there are formidable problems due to the long generation cycle and large plant size, resulting in increased labor and space. An alternative approach is the integration of plant biotechnology including tissue culture and molecular biology into traditional breeding programs, which has the potential to overcome the limitations of decreased rate of genetic progress. Establishment of *in vitro* regeneration of adventitious shoots from the *L. chinense* would be useful for genetic improvement, including rapid propagation and genetic transformation. However, there are a few reports related to clonal propagation and regeneration from *L. chinense* (Hu et al. 2002; Jang et al. 1998).

Construction of homogeneous population with desirable characters for industrial purposes is very important, especially for medicinal plants. Many attempts to propagate valuable plants on a large scale through *in vitro* system have been successful however, the homogeneity of such populations has not been tested before planting for mass propagation.

The probability of spontaneous mutations in *in vitro* culture is inevitable because of cultural environments,

nutrients and/or plant growth stimulants (Scrowfort 1984). Most spontaneous variants can be regarded as silent if morphologies and other physiological traits are identical to the normal plants. The somaclonal variation can be reduced if *in vitro* plants can be substituted as clonal propagules. *In vitro* propagation through regeneration via organogenesis or somatic embryogenesis leads to increased somaclonal variation (Bailey et al. 1983; Chin et al. 1998; Gamborg et al. 1977; Ogura, 1990). The true-to-type propagation through various tissue culture pathways implies that the least or no genetic variation occurs in the subsequent generation. Early detection and elimination of such silent mutations can facilitate to establish efficient and reliable homogeneous true-to-type population of *in vitro* regenerants. The RAPD analysis, among other molecular techniques, has been widely used for detecting genetic variations of the silent mutants (Roy et al. 1992).

Our objectives in this research were to optimize adventitious shoot regeneration protocols from leaf sections of *L. chinense* and examine silent somaclones with RAPD methods. This would provide information about the frequency of spontaneous mutation *in vitro* and assess the level of background genetic changes resulting from adventitious pathway.

Materials and Methods

Plant materials

A cultivar of *L. chinense* was obtained from the Forest Research Institution (FRI), South Korea and used for proliferating *in vitro* stocks. Stems from plants grown in the pots were cut into 2 cm each and sterilized with commercial clorox (5.25% active gradient) for 10 min. Excised stem segments were horizontally cultured on MS basal medium supplemented with 0.2 mg/L BA. *In vitro* shoots were subcultured on the same medium every 4 weeks and the cultures were incubated in the glass jars containing MS (Murashige and Skoog 1962) medium without PGR (Plant Growth Regulators) to elongate shoots and expand leaves.

Fully expanded leaves (1.5 cm in length and 1 cm in width) from *in vitro* cultures were excised from the mid region of growing shoots and petiol parts were removed. Leaves were then dissected into halves along the mid-veins, but excluding them, and punctured five times with needles. Explants were placed on the Petri dishes containing medium with different basal formulations and cytokinins, and ensuring that the abaxial surface in contact with medium.

Preparation of medium and culture maintenance

All media contained 100 mg/L myo-inositol, and various types and concentrations of cytokinins. The pH of the medium was adjusted with 0.5 N KOH and 0.5 N HCl before autoclaving and the medium solidified with 4 g/L of Difco bacto-agar and 1.5 g/L of the phytagel (Sigma) after autoclaving (1.1kg cm^{-2} at 121℃ for 15 min). Plates were wrapped with parafilm and incubated in a culture room at 23℃ under a 16 h photoperiod at 60 to 70 $\mu molm^{-2}s^{-1}$ provided by cool-white fluorescent tubes.

Regeneration responses in different media

To determine regeneration efficiency of *L. chinensis*, eight media were tested; Gamborg B5 (Gamborg et al. 1968), GD (Gresshoff and Doy 1972), 1/2 MS (Murashige and Skoog 1962), full-strength MS, 3/2MS, N & N, SH (Schenk and Hilderbrandt 1972), and WPM (McCown et al. 1980). The B5, 1/2MS, MS, 3/2MS, N&N and SH media contained 2 mg/L of glycin, 0.5 mg/L of nicotinic acid, 0.5 mg/L of pyridoxin.HCl, and 0.1 mg/L thiamine.HCl, while GD contained 0.2 mg/L D-biotin, 4.0 mg/L of glycone, 0.1 mg/L of nicotinic acid, 0.1 mg/L pyridoxin.HCl and 1.0 mg/L thiamine.HCl. WPM medium contained 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine.HCl, and 1 mg/L thiamine.HCl. All media contained 10 μM zeatin. Zeatin was sterilized with filter (0.2 μm in pore size) before adding to the medium. All cultures were incuabted under the dark condition for 3 weeks followed by light treatment as described in above.

Effect of cytokinins on induction of adventitious organs

Based on the results of the first experiment, the best meduim was selected and used to determine the best cytokinin and its concentration. The four cytokinins (2ip: 6-γ-γ-dimethylallylamino purine, BA: benzyladenine, kinetin and zeatin) at six concentrations (1, 5, 10, 10, 30 and 40 μM) with control were examined for this experiment. All cyotkinins except zeatin were added before autoclaving. Zeatin was sterilized with filter and added to cooled medium after autoclaving.

Detection of somaclonal variants using RAPD

From regenerants, 50 to 100 mg leaf materials were ground in liquid nitrogen. Five hundred μl of 2xCTAB (hexadecyltrimethylammonium-bromide) solution was added and incubated at 65℃ for 5 min prior to the addition of the same amount of chloroform/isoamyl alcohol. The mixture

was then vortexed for 10 sec followed by a spin of 10 sec by pulsing it (Sul and Korban 1996). The aqueous layer was transferred to the new tubes and two volumes of ice cold 95% ethanol was added, mixed by swiring it gently. White colored fiber was formed and the tube was centrifuged for 10 to 15 sec and the white pellet was collected. After decanting the ethanol, the genomic DNA samples were washed twice with 70% ethanol. The ethanol was then removed by pippeting and the tubes were transferred to a speed-vaccum (Hetovac VR-1, Denmark) for drying (2-3 min) or dried at room temperature. 100 μL of TE buffer was added after complete drying, and the tubes were incubated at 65℃ for 5 to 10 min to dissolve genomic DNA, and Rnase was then added. All samples from different regenerants along with the undigested lambda DNA (0.5 μg) were electrophoresed to measure amounts and diluted with TE buffer. All DNA samples were stored at the -20℃ until used.

PCR reactions were carried out in 25 μL volume per reaction, containing 1 μL 50 ng genomic DNA, 2.5 μL (10 mM dNTP), 1 μl random primer (25 pmole), 2.5 μL 10x buffer, 1 μL Taq polymerase (2.5 U/μL). Each reaction was overlaid with three drops of mineral oil to prevent evaporation. PCR reactions were carried out in Techne in a GENUS thermal cycler (England) under the following programme conditions: 94℃ for 5 min at initial step, 35 cycles at 92℃ for 45 sec, 36℃ for 45 sec and 72℃ for 1 min, followed by one cycle at 72℃ for 5 min. Random 10-mer primers (F-01 to F-15) were obtained from OPERON (Almeda, California). Amplified products were then resolved on a 1.6% agarose gel in 0.5x TBE buffer, stained with ethidium bromide and visualized by illumination with ultraviolet light (312 nm).

Statistical analysis

For each treatment, 10 leaf sections were cultured on the Petri-dishes and three plates were used for each treatment. Each plate was regarded as individual replicate and used for statistical analysis. For all experiments, data were collected on number of adventitious shoots after eight weeks of *in vitro* culture. For statistical analysis, per cent data were transformed into arcsine values and anlayzed by ANOVA; means were subjected to LSD and ANOVA tests using SAS statistical package (SAS Institute Inc. 1985).

Results

Within a week after dark treatment, most leaf sections started to expand and swell over wounded areas leading to development of callus. The callus continued to grow up to 3 weeks until transferred to light where white-yellowish callus changed into greenish color gradually. Eventually greenish organogenic callus produced a number of adventitious organs including roots and/or shoots. All shoots and roots differentiated from the intervening callus and were visible after 1 week of light culture but most adventitious organs developed after 2 weeks.

Regeneration response in different media

ANOVA showed significant interactions ($P \leq 0.01$) among different media on regeneration frequency and number of shoots. The frequency of regeneration and number of adventitious shoots in response to media are shown in Table 1. Although all media induced adventitious shoots, the highest regeneration frequency was observed on WPM (86.7%) compared with 1/2MS (80%) and MS (75%). However, the highest number of adventitious shoots were produced on MS (5.1), followed by 1/2MS (4.7), WPM (3.5) and B5 (3.3) (Figure 1 and Table 1). The highest regeneration frequency did not imply the production of the highest number of shoots (Table 1). Even though WPM showed the highest regeneration frequency, the average number of shoots was lower than those of 1/2MS and MS media (Table 1). Similarly, MS produced the highest number of shoots but had lower regeneration frequency than those of WPM and 1/2MS.

The 1/2MS showing the optimal efficiency in regeneration frequency and number of shoots was selected for further regeneration experiment.

Table 1. Influence of different media types on regeneration frequency and average number of shoots from *L. chinensis* leaf section. Each medium was supplemented with 10 μM zeatin and data were collected after 8 weeks in culture.

Types of medium	Regeneration	
	Frequency (%) ± SE	No. shoots/ explants +SE
B5	66.7 ± 3.3 bcd[a]	3.3 ± 0.3 ab
GD	43.4 ± 3.3 d	1.0 ± 0.1 c
1/2MS	80.0 ± 0 ab	4.7 ± 0.3 a
MS	75.0 ± 3.5 abc	5.1 ± 0.2 a
3/2MS	43.3 ± 1.7 d	1.5 ± 0.3 bc
N & N	40.0 ± 8.7 d	1.9 ± 0.5 bc
SH	46.7 ± 1.7 cd	2.0 ± 0.2 bc
WPM	86.7 ± 4.4 a	3.5 ± 0.6 ab

[a]Means of each column followed by the same letter are not significantly different at $p \leq 0.05$ level, according to LSD test.

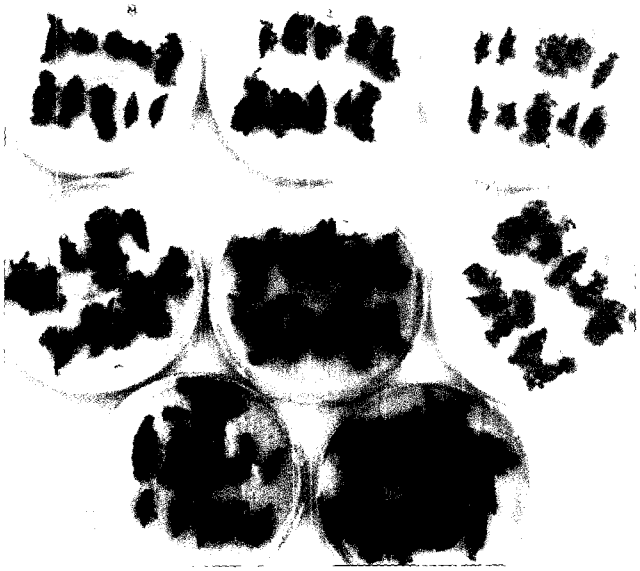

Figure 1. Induction of adventitious shoots from *Lycium chinense* leaves. Shoots were induced after 6 weeks on each subculture medium. Top left: GD, NN, SH; Middle left: B5, 1/2MS, WPM; Bottom left: 3/2MS, MS

Effect of cytokinins on induction of adventitious organs

Three cytokinins (BA, kinetin and 2ip) failed to induce adventitious organs from the leaf sections. Most explants in culture showed brownish color resulting in the death of all explants. Some leaf sections on medium containing kinetin proudced calli but did not induce adventitious organs (Figure 2). All leaf sections cultured on medium containing zeatin produced healthy calli and subsequently adventitious shoots even though the lowest concentration (5 μM) failed to induce organs (Figure 2). Significant interactions ($P \leq 0.01$) were observed among zeatin concentrations on regeneration frequency and average shoot number. The frequency of regeneration and average number of shoots in response to different zeatin concentrations is presented in the Table 2. Interestingly, the control (1/2 MS without zeatin) produced

Table 2. Influence of zeatin concentrations on regeneration frequency and average number of shoots from *L. chinense* leaf sections. Data was collected after 8 weeks in culture.

Zeatin concentrations (μM)	Regeneration	
	Frequency (%) ± SE	Ave. no. shoots/ explants +SE
Control	46.7 ± 8.2 a[a]	1.4 ± 0.3 cd
1	0.0 ± 0.0 c	0.0 ± 0.0 d
5	13.3 ± 1.9 c	0.1 ± 0.0 cd
10	56.7 ± 6.1 b	3.3 ± 0.6 b
20	100 a	9.0 ± 0.8 a
30	93.7 ± 4.6 a	9.4 ± 0.8 a
40	35.0 ± 2.5 bc	1.8 ± 0.2 bc

[a]Means of each column followed by the same letter are not significant different at 0.05 level, according to LSD tests.

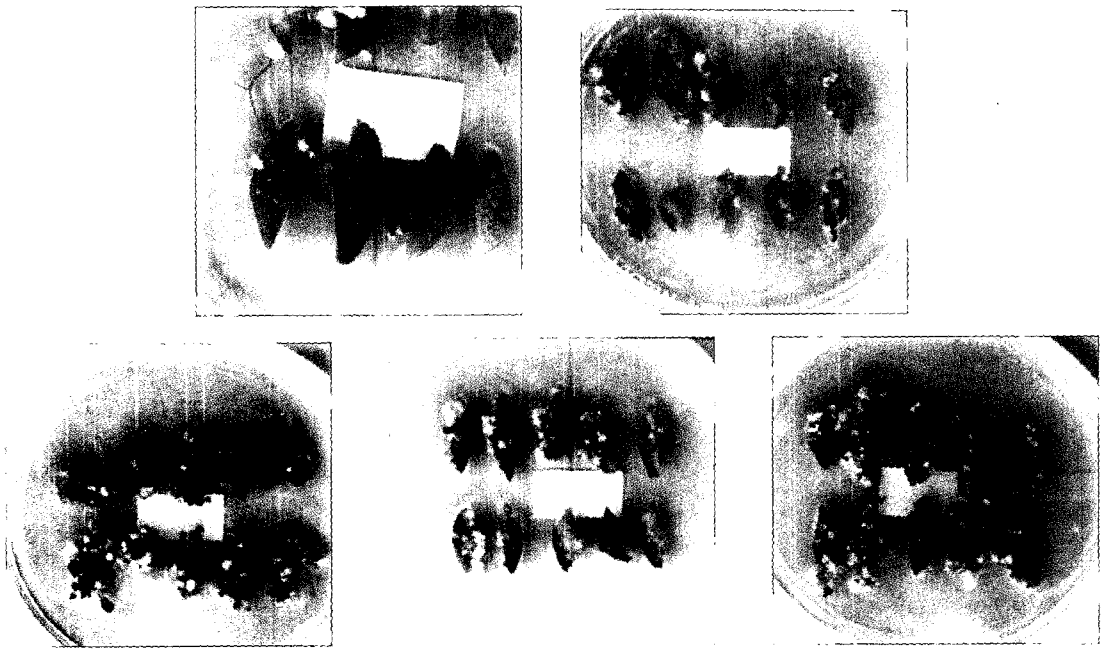

Figure 2. Induction of adventitious shoots of *Lycium chinense* leaves after 6 weeks of culture on 1/2MS medium supplemented with various concentrations of zeatin.

adventitious shoots and roots (44.2% regeneration frequency), however, the number of spontaneous shoots was very low (1.8).

Each organ differentiated from discernable different locations excluding the possibility of somatic embryos. The highest regeneration frequency (100%) was observed in 20 μM zeatin, followed by 93.7% in 30 μM zeatin, with corresponding number of 9 and 9.4 shoots. Overall regeneration frequency as well as number of shoots continuously increased up to 30 μM zeatin and decreased dramatically at 40 μM. To obtain complete plantlets *in vitro*, each regenerant separated from the clumps of shoots was transferred to 1/4 MS without any growth stimulants. Within 2 weeks, shoots were elongated and leaves expanded. Spontaneous rooting occurred on all subcultured regenerants.

Detection of somaclonal variants using RAPD

To examine somaclonal variants, 15 random primers from operon (OPF 1 – 15) were tested to assess the RAPD variation. A total of 40 regenerants, showing the identical normal phenotype, were randomly taken for genomic DNA isolation. The DNA banding patterns were examined using UV-transilluninator and number of bands were scorded. Fourteen primers produced the identical banding patterns, however, bands amplified from OPF-4 primer showed polymorphisms in two regenerants (Figure 3). The two bands of (approximately 1 kb and 1.5 kb) were missing in two regenerants. To confirm RAPD polymorphisms, genomic DNA was reisolated from the two regenerants as well as three other regenerants and amplified with the same primer (Figure 4). Somaclone (#10) had two bands like other regenerants, however, clone (#11) did not detect, the missing bands. Thus, this somaclone is the normal phenotypes but with silent DNA variation.

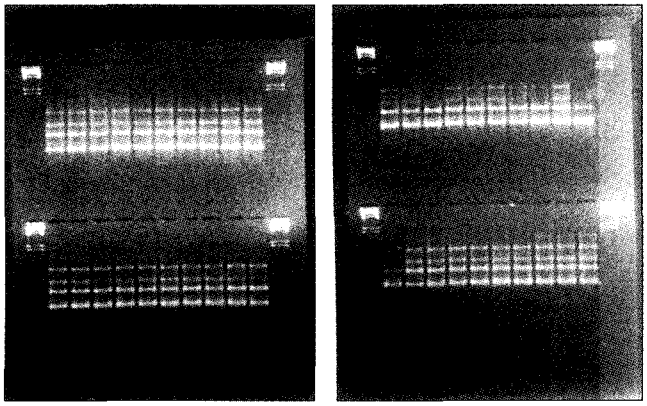

Figure 3. Detection of polymorphic bands in 40 plantlets of *Lycium chinense* regenerated via callus using random primers (OPF-4). Arrows indicate polymorphic bands.

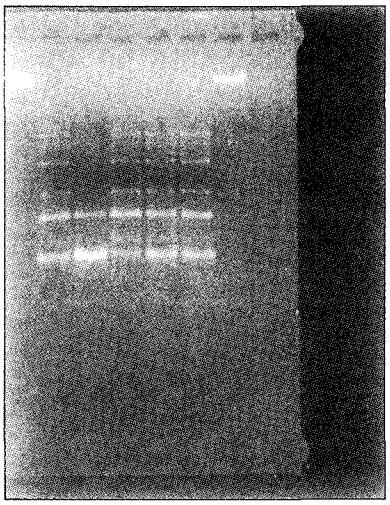

Figure 4. Second RAPD PCR amplification of *Lycium chinense*. Polymorphic bands as revealed by gel electrophoresis of RAPD fragments generated by primer (OPF-4).

Discussion

The adventitious shoot regeneration *in vitro* is possible from *L. chinense*. All of adventitious shoots were regenerated from calli from wounding sites like observed in other plant regeneration systems (Tibok et al. 1995; Lee et al. 1997).

Basal medium compositions clearly influenced plant organogenesis as different plants have different requirement of medium, ionic strength and sometimes carbon sources (Bon et al. 1998; Konan et al. 1997; Kaneda et al. 1997; Sul and Korban 1998). Moreover different explants derived from the same plant require different basal media compositions and growth regulators (Laparra et al. 1997). Like other plants, all the organogenic traits observed for L. chinense were significantly influenced by different medium formulations (Table 1 and Figure 1). Eight types of media consisted macroelements and total ionic concentration, different from one another. Three media (WPM, 1/2MS and MS in order) showed the best regeneration frequency, however, MS, 1/2MS and WPM in order produced higher average number of shoots. MS medium has the highest ionic strength than other media, while WPM and 1/2MS have about half of the ionic strength than that of MS. Since there were no significant differences among MS, 1/2MS and WPM media, we concluded that *L. chinense* leaves as explants were not influenced by the total ionic strength but by specific medium ingredients for inducing adventitious organs. This may lead to the conclusion that lycium leaves are sensitive to the ingredients of the tested medium but not influenced by

diverse range of ionic strengths. For further regeneration experiment, 1/2MS medium was selected because the number of shoots was higher than in other two media.

Like other plants (*P. deltoids*), *L. chinense* showed cytokinin toxicity (sensitivity) because all cultured leaf sections except those in zeatin turned brown and later died. Since zeatin is one of the natural plant hormone, it is believed to have the least side-effect on plant when used in *in vitro* culture as verified in other plant species (*P. deltodes*, *Vigna unguiculta* L. Walp.) (Pellegrineschi 1997). Kinetin produced calli from the wounding sites to some extent but no regeneration was observed and all calli were dead after subculturing. We concluded that growth regulators strongly influenced the organogenesis of *L. chinense* explants. Adventitious organs (shoots and roots) appeared on the explants on medium with no PGR (control), suggesting that endogeneous hormone levels in the *L. chinense* may be high enough to induce adventitious organs (Figure 2). However, the frequency of regeneration and the number of adventitious organs were minimal and not enough for mass propagation. Zeatin concentrations at 20 to 30 μM led to over 96% regeneration frequency and over 9 shoots per explants. More than 30 μM zeatin decreased the regeneration frequency and average number of shoots (Table 2). There was no significant difference between 20 to 30 μM zeatin. Thus, 20 to 30 μM of zeatin is the optimum for inducing the highest regeneration from the *L. chinense* leaf segments. However, we recommend a lower concentration of zeatin, especially for the purpose of mass propagation, because of higher cost of this hormone.

The regeneration system passed through a callus phase, even if zeatin was used as cytokinin for inducing adventitious organs, thereby increasing possibility of inducing somaclonal variation. Therefore, the regenerated plants should be observed for parental or clonal traits. In most cases, regenerated plants are examined based on phenotypes. However, this system cannot discriminate silent mutations, in which expression of mutated gene appears at the late developmental stages. The use of RFLP and RAPD methods can detect variation at early stage to facilitate screening of somatic mutants (Gesteira et al. 2002; Hashmi et al. 1997; Chen et al. 1998). The RAPD has strong advantages over the RFLP because of simple procedure and easy handlings. In our study, only one regenerant from one of the 15 primers (one out of 600 tests in total) showed polymorphic bands. However, this regenerant has normal phenotype. In case of Panax, no somatic variants were detected from regenerated plants through somatic embryogenesis (Shoyama et al. 1997). Mussio et al. (1998) reported that minor somatic variation in regenerants, derived from immature leaf lobes of cassava plants, was detected. Our results are consistent with those of Mussio el al. (1988) in that minor incosistency of DNA variation can be neglected and most regenerants can be substituted for true-to-type propagules.

An efficient, rapid and reproducible system for regenerating adventitious shoots *in vitro* from leaf sections of *L. chinense* was developed. With RAPD analysis, regenerants showing genetic stablility and same phenotype when compared with normal plants. Thus, this protocol can be substitued for mircopropagation *in vitro* and used for genetic transformation via either Biolistc or *Agrobacterium*-mediated transformation studies.

Acknowlegement

This work was supported by Korea Research Foundation Grant (KRF-2001-003-G00017)

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