
Application of *in vitro* conservation in *Vetiveria zizanioides* Nash

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The shoot tip of *Vetiveria zizanioides* Nash var. Songkla 3 was investigated to conserve by slowly growth conditions. After 12 months of preservation shoot forming percentage, number of shoot, shoot length and genetic fidelity were evaluated. Shoot tips were excised and cultured on various strengths of basal Murashige and Skoog (MS) medium supplemented with paclobutrazol (PBZ). The cultures were placed under light condition at 14 h photoperiod, 27 ± 1 °C for 12 months. Results revealed that reducing strength of MS to one-fourth (1/4) of original concentration supplemented with 3 mg/l PBZ gave the highest percentage of shoot formation and number of shoot at 75% and 3.76 shoots per explant, respectively. The shoot length was 2-6 mm after 12 months. Random amplified polymorphic DNAs (RAPDs) marker revealed that there was no variation of DNA profiles between non-conserved and conserved plants and among conserved plants. These results showed that *in vitro* conservation by reducing strength of culture medium with PBZ was a practical method for the medium-term storage of vetiver grass.

Key words: vetiver grass, *in vitro* conservation, shoot tip, RAPD

Introduction

Vetiver grass (*Vetiveria zizanioides*) Nash is generally used as protection against soil erosion and loss of moisture (Vietmeyer and Ruskin, 1993). Moreover, the roots contain an essential oil consisting of more than 150 sesquiterpenoids, which is used as a component for perfumes, scenting soaps and as a fixative to prevent the evaporation of volatile oils. The seed germination of vetiver is usually low, and led to the difficulty in propagation of the species by sexual method (Vietmeyer and Ruskin, 1993). Vetiver grass is amenable to tissue culture. Propagation systems have been developed that are optimal for a wide range of cultivars. Plant regeneration has been achieved from inflorescence tissue (Nanakorn *et al.*, 1998), leaf explant (Mathur *et al.*, 1989; Mucciarelli *et al.*, 1993; Leupin *et al.*, 2000) and crown (Leupin *et al.*, 2000). In addition, it seems to be a relatively stable crop, both in the field, and in tissue culture, so is suited to *in vitro*

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storage. As vetiver grass is a vegetatively propagated crop, it is traditionally conserved in field collections. This is not an effective conservation strategy for medium to long term preservation of genetic resources. *In vitro* germplasm conservation presents different advantages such as: (1) free from genetic erosion, (2) the possibility for the establishment of core collection with long term genebanks, (3) free from fungi and bacteria, (4) not expensive, when *in vitro* facilities are already present, (5) easy and convenient for international distribution. (Malaurie *et al.*, 1998). A recent status report on the use of *in vitro* techniques for the conservation and use of plant genetic resources concluded that slow growth techniques are now successfully and routinely applied to a range of species and across a range of genotypes within species. The use of these techniques allows storage of healthy germplasm with extended subculture intervals, thus reducing time and costs for maintenance. The application of slow growth storage technique is culturing in the reducing strength of basal medium or supplementing the culture medium with growth retardant, temperature reduction, often combined with a decrease in light intensity or culture in the dark. The Pacific Regional Agricultural Programme, demonstrated that temperature reduction was the most practical method for slowing down the growth rate of taro (*Colocasia esculenta*). Taro could be maintained at 20°C, for 9 to 12 months, without subculturing, depending on the variety (Taylor *et al.*, 2001). Moreover, there are reports in the literature of taro being stored for more than eight years at 9°C in total darkness, with transfer intervals of approximately three years. The reported that taro could be conserved for three years at 9°C, and still be viable. This pilot study was a model to carried out shoot tip of vetiver grass variety Songkla 3 growing under slow growth conditions, by culturing in reducing strengths of basal MS medium with various concentrations of paclobutrazol (PBZ) under standard of culture temperature or low temperature.

Materials and methods

Plant materials

In vitro stock plants of vetiver grass variety Songkla 3 were obtained by culturing 5-10 mm long shoot tips, isolated from sterilized field-grown shoots at Plant Science department landscape area, in liquid MS medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA and 3% sucrose at pH 5.7 in 250 ml cultured bottle. Each bottle containing with 30 ml of culture medium was cultured by shaken at 100 rpm on a rotary shaker and subculture at 2 months intervals. To study application *in vitro* conservation, shoot tips were excised about 2-3 mm and culture on slow growth condition medium.

Study of slow growth condition

Shoot tips were culture in glass bottle (8 oz size) containing 25 ml of MS medium at various strengths (1/4MS, 1/2MS, 3/4MS or 1MS) supplemented with PBZ at various concentrations (0, 0.25, 0.5, 1.0 mg/l) for pre-test growth condition (2 months). For medium term (12 months) conservation, more investigation in 1/4MS with 2, 3 and 4 mg/l PBZ were carried out. All culture media were solidified with 0.75% agar, adjusted pH to 5.7 with 0.1 N HCl before adding agar and autoclaved at 1.05 kg/cm², 121°C for 15 min. The cultures were either placed under light conditions at 1,300 lux illumination for 14 h photoperiod, 27±1°C or low light intensity in refrigerator controlled temperature at 4±1°C. Completely randomized design (CRD) with 5 replicates (each replicate consists of 8 shoot tips) was designed. The data on percentage of shoot formation, the number of shoot per explant were recorded from 2 to 12 months. The genetic fidelity of *in vitro* conserved plants was analyzed by random amplified polymorphic DNA (RAPD) as following procedures.

DNA analysis

DNA extraction and purification

Total genomic DNA was extracted from leaves of control and *in vitro* conserved shoots following the standard CTAB method with minor modifications. A 100 milligrams of leaves were ground in the presence of LN, then homogenized in 750 µl of extraction buffer (2% CTAB, 0.5M Na-EDTA pH 8.0, 1% PVP, 8.12% NaCl, 1M Tris-HCl pH 8.0 and 2% β-mercaptoethanol and incubated at 60°C for 1 h. The supernatant was twice extracted with chloroform (24:1 v/v). The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 20-50 µl of TE buffer (1 M Tris-HCl pH 7.5 and 0.25 M Na-EDTA pH 7.0) and stored at -20°C. The DNA concentration was determined electrophoretically using λ DNA as standard.

RAPD analysis

Seven random primers were selected for analysis of DNA template from the control and *in vitro* conserved shoots. The primers were OPJ4, OPN15, OPAB01, OPAB09, OPAB14, OPR3 and OPR12. These arbitrary primers purchased from Operon Technologies, Alameda, USA. were employed for DNA amplification. The 25 µl reaction mixtures contained about 20 ng of template DNA, 0.2 µM of primer, 2.5 mM of the dNTPs and 0.2 µl *Taq* DNA polymerase in manufacturer's buffer. DNA was amplified on a BIOER XP cycle programmed as follows: a preliminary 2 min denaturation at 94 °C; 35

cycles of 1 min at 94 °C (denaturation), 1 min at 37 °C (annealing), and 2 min at 72 °C (extension); and a final extension at 72 °C for 5 min. The amplified products were resolved on 1.5% agarose electrophoresis using 0.5xTris–acetic acid–EDTA buffer by electrophoresis at 100 V for 1.5 h and stained with ethidium bromide (10 mg/ml). The image bands were acquired through UV light using Bio-Imaging Gel documentation system. The molecular size of the amplicons was determined with reference to the DNA ladder 100 bp.

Results and discussion

Study of slow growth condition

The aim of the conservation of plant genetic resources was not only to store germplasm but also to minimize variations to the lowest variable level during conservation. Any conservation method can be used only if the plant material remains genetically stable. Analysis of genetic variation mainly involves growth and development, physiological functions and genetic markers. The morphological markers studied in the following aspects: regrowth rate, shoot length, leaf shape, leaf width/length ratio, and root length. In this study, we found that shoot tips cultured on reducing strength of culture media with higher concentration of PBZ gave the ratio of shoot formation, number of shoot higher than normal culture medium and low concentration of PBZ. One-fourth (1/4) MS medium supplemented with 3 mg/l PBZ gave the highest shoot formation and number of shoots at 75% and 3.76 shoots/explant, respectively. Shoot length obtained in this culture medium was shorter at 2-6 mm after 1 year of culture. Reducing strength of culture medium without PBZ was not suitable for reducing growth (Table 1, Fig. 1). As *in vitro* conserved shoots were differ from control shoot, which caused by the effect of slow growth condition in cultures. So, morphological markers were inefficient for detection of variation. Molecular markers are of great important in this case to examine genetic variation at the DNA level. RAPD and AFLP are the two most widely used molecular markers. They are highly polymorphic and can cover the whole genome. RAPD is an easily performed molecular technique and the differences in RAPD fragments denote alterations of the genome. So it is a practical way to analyze genetic variation with select reaction parameters (Liu *et al.*, 2004). Zhang *et al.* (2001) tested plantlets regenerated from shoot tips of *Amorphophallus* cryopreserved by vitrification using RAPD markers and did not find any DNA alteration. In another study, adventitious buds of haploid rice were successfully cryopreserved and subsequently regenerated surviving shoots. RAPD markers showed no genetic variation (Zhang and Hu, 2000). Similar results were also obtained from slow growth and cryopreservation of the potato (Harding, 1991). From 7 primers of RAPD marker used in these study, 3 primers gave the uniform DNA band, A total of 144 electrophoresis bands produced, there were no differences bands found in comparison with the

controls (Fig. 2). However, about 21 *in vitro* conserved shoots (from 7 treatments) represent a small population, which is a potential limitation in the assessment of genetic fidelity in the present study. At the same time, the RAPD technique has some flaws such as variable replications and lack of stability, and is easily affected by some unknown factors. Although no different RAPD bands have been detected between individual shoot in some treatment that could produce DNA content (data not show), this does not provide final proof that no variation occurred in the *in vitro* conserved shoots in comparison with the controls. A more thorough tests including simple sequence repeat (SSR), AFLP, sequencing analysis, particularly of more variable regions, or a greater sampling of the genome need to be performed.

Table 1. Effect of PBZ and strength of basal MS medium on shoot formation percentage and number of shoots and shoot length after conserved for 12 months.

PBZ (mg/l)	Concentration of basal medium	Shoot formation (%)	No. shoot/explant	Shoot length (mm)
0	1/4MS	62.5	1.0	approx. 60
	1/2MS	25.0	1.0	reach to lid
	3/4MS	6.25	1.0	reach to lid
	1MS	D	D	reach to lid
0.25	1/4MS	56.25	1.3	5-12
	1/2MS	ND	ND	ND
	3/4MS	D	D	ND
	1MS	D	D	ND
0.5	1/4MS	ND	ND	ND
	1/2MS	ND	ND	ND
	3/4MS	54.17	2.06	2-10
	1MS	ND	ND	ND
1.0	1/4MS	ND	ND	ND
	1/2MS	62.5	2.2	2-5
	3/4MS	ND	ND	ND
	1MS	ND	ND	ND
2.0	1/4MS	50.0	3.25	3-7
3.0	1/4MS	75.0	3.76	2-6
4.0	1/4MS	62.5	2.88	2-4

ND = not detected.

D = browning and dead.

Conclusion

In conclusion, we did not find genetic changes in the *in vitro* conserved shoots using RAPD marker. So, culturing shoot tips in reduction strength of basal medium (1/4 MS) with PBZ (3 mg/l) is a practical method for the medium-term storage (12 months) of vetiver grass germplasm. For further study it should be prolonged the culturing time (24-36 months) and increased the number of replication and number of plant materials in replication as well.

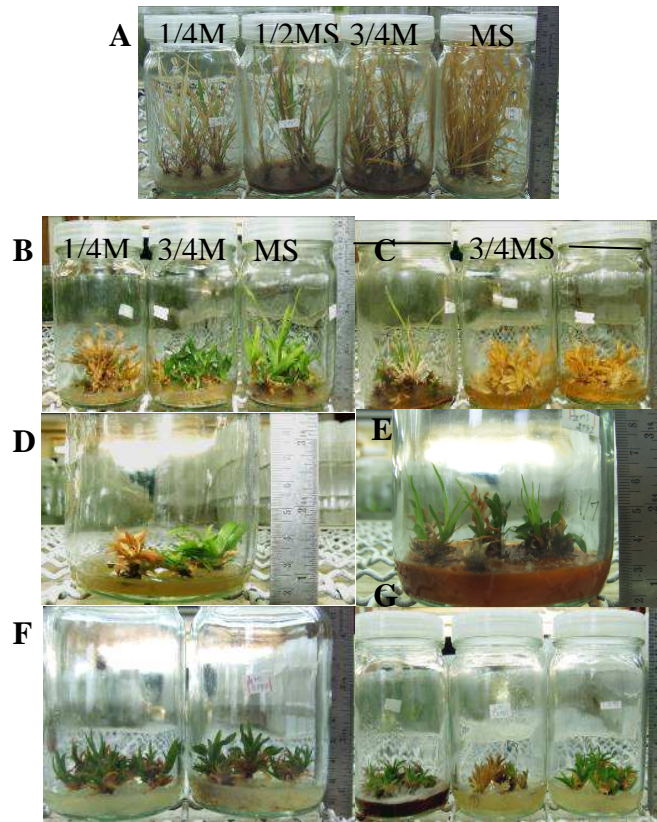


Fig. 1. Shoots formation from *in vitro* conservation after 12 months of culture on various strengths of basal MS medium with PBZ. (A) 0 mg/l PBZ, (B) 0.25 mg/l PBZ, (C) 3/4MS with 0.5 mg/l PBZ, (D) 1/2MS with 1 mg/l PBZ, (E) 1/4MS with 2 mg/l PBZ, (F) 1/4MS with 3 mg/l PBZ, (G) 1/4MS with 4 mg/l PBZ.

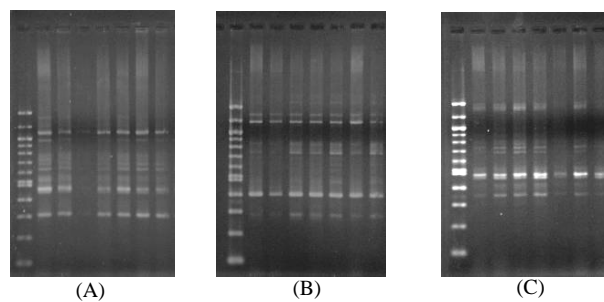


Fig. 2. Molecular analysis of *Vetiveria zizanioides* Nash *in vitro* conservation using RAPD. Electrophoretic analysis of DNA amplification with OPJ4 primer(A), OPN15(B) and OPAB09(C). Lanes designated as M represents 100 bp molecular weight marker, Lane number 1 corresponds to the control shoot, the others are *in vitro* conserved shoots.

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