THE *IN VITRO* REGENERATION OF VETIVER (*Vetiveria zizanioides* (L.) Nash) USING THIN CELL LAYER CULTURE OF INFLORESCENES and SELECTION FOR SALT TOLERANT CALLUS CLONES

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ABSTRACT

A new and pretty simple method was designed to in vitro regenerate and select the salt tolerant callus clones of unfertile species, Vetiveria zizanioides (L.) Nash. The transverse thin cell layers (tTCLs) excised from the axis of inflorescenes placed on MS (Murashige and Skoog, 1962) medium supplemented with 2 mgL^{-1} NAA formed calli which produced the bud clusters after transferred to MS medium containing 1 mgL^{-1} BA. Somatic embryos derived from tTCLs on MS medium containing a combination of 2 mgL^{-1} NAA and 1 mgL^{-1} BA. Both bud clusters and somatic embryos were later transferred to MS without growth regulators, where thousands of invitro plantlets formed and developed. After subcultured 5 times on the medium supplemented with gradually increasing concentration of NaCl (0.5-2.5%), 8.89% of callus clones survived and formed high salt tolerant Vetiver plantlets.

Key words: *Vetiveria zizanioides* (L.) Nash – invitro regeneration – salt tolerant callus clones

Abbreviations: BAP, 6-benzylaminopurine – 2,4-D, 2,4-dichlorophenoxyacetic acid – NAA, α-naphthalenacetic acid – MS, Murashige and Skoog (1962) - tTCL, tranverse Thin Cell Layer

INTRODUCTION

Vetiver, *Vetiveria zizanioides* (L.) Nash, *Poaceae* is a perennial tropical grass. Vetiver grows in large, densely tufted clumps from a stout, compact rhizome (crown) with erect clumps up to 3 meters high and it roots bind the soil beneath the plant, reaching depths of up to 4 meters. So, nowadays , more than 160 countries use vetiver hedges for protecting against erosion. Moreover, it is a pretty effective species for phytoremendiation methods of polluted areas. The essential oil extracted from its roots is also used in medicine and cosmetic (Ruth Elisabeth Leupin, 2001). Since most of its varieties are unfertile, *in vitro* culture of Vetiver which has been studied recently is rapid propagation. Besides, it is also effeccient to obtain and select the variants. Immature or young inflorescences are an important explant for initiating tissue cultures of *Poaceae*, especially the grass species (Bui van Le, 1997; K. S. Alexandrova, P. D. Denchev and B. V. Conger, 1996). Keshavachadran et al. (1997) and Sreenath and Jagdshehandra (1990) reported that calli and somatic embryos were derived from immature inflorescences of Vetiver grass.

The objective of this research were to study the in vitro culture of Vetiver zizanioides L. Nash from Australia by means of callus or somatic embryo from inflorescence then primarily find the proceduce to select the salt tolerant callus clones, which was successful in rice (Mori Koh-Ichi and Kinoshita Toshiro, 1990), obtain high salt-tolerant Vetiver plantlets.

MATERIALS AND METHODS

Plant materials

7-10 day-old inflorescenes covered by leaves completely were collected from Forestry and Agriculture university, Ho Chi Minh city, Vietnam. The sterile inflorescences were obtained after the cover leaves were surface sterilized by heat .

Tranverse thin cell layers (tTCL s) were excised from the axis of inflorescences (0,5-1 mm thick from the bottom 5 mm portion).

In vitro regeneration

The explants were placed on basic medium [MS medium (Murashige and Skoog, 1962) + 3% sucrose + 0.7% agar-agar] supplemented with different kind or/ and ratio of auxin (1-5 mgL^{-1} NAA or 1-5 mgL^{-1} 2,4-D) to form callus or somatic embryo when combined with cytokinin (0.5–2.0 mgL^{-1} BA). Calli were transfered to medium supplemented 0.5-2.0 mgL^{-1} BA to form shoots.

Both regenerated shoots and somatic embryos formed from the tTCLs were transfered to growth regulator free basic medium for vigorous and phenotypically normal shoot development and rooting. Young regenerated plants were then transfered to the nursery after 2 weeks.Selecting the salt tolerant callus clones

Several callus lines obtained from tTCLs of young inflorescenes were placed on basic medium (MS) supplemented with 2 mgL^{-1} NAA and NaCl (NaCl concentration increasing from 0.5% to 1.0%, 1.5%, 2.0% and 2.5% for one subculture). They were subcultured every week.

The calli surving after the selection were tranferred to basic medium supplemented with optimal concentration of BA for regenerating shoot and 2.5% NaCl. Shoots then developed and rooted on basic medium supplemented 2.5% NaCl. Regenerated plants were planted in nursery after two weeks.

Data collection and analysis

Approximately 50 tTCLs were used for each experiment. Observation were carried out once a week. The result were scored as:

- The average percentage of tTCLs forming callus or somatic embryo and calli surving and forming shoots.
- The average numbers of somatic embryos and shoots per tTCL

The average number ± standard deviation (SD) corresponds to the mean value of at least 3 replications.

RESULTS AND DISCUSSION

In vitro regeneration

Two kind of *in vitro* developing ways in *Vetiver zizanioides* were observed in this research. One was organogenesis and the other, somatic embryogenesis that was denpended upon kind or dosage of the plant growth regulator in the medium.

Although tTCLs cultured on MS medium with 1 mgL^{-1} 2,4-D or 2 mgL^{-1} NAA produced calli vigorously after 2 weeks (Fig. 1), only those formed on the medium contained NAA took form the bud clusters on MS medium with 1 mg L^{-1} BA after 7 days (345±9.55 buds per tTCL of young inflorescene) (Fig. 2). The planlets were obtained after the bud clusters subcultured to MS free growth regulators for normal development of shoots and rooting.

Fig 1: Effect of 2,4-D and NAA on the percentage of TCLs forming calli

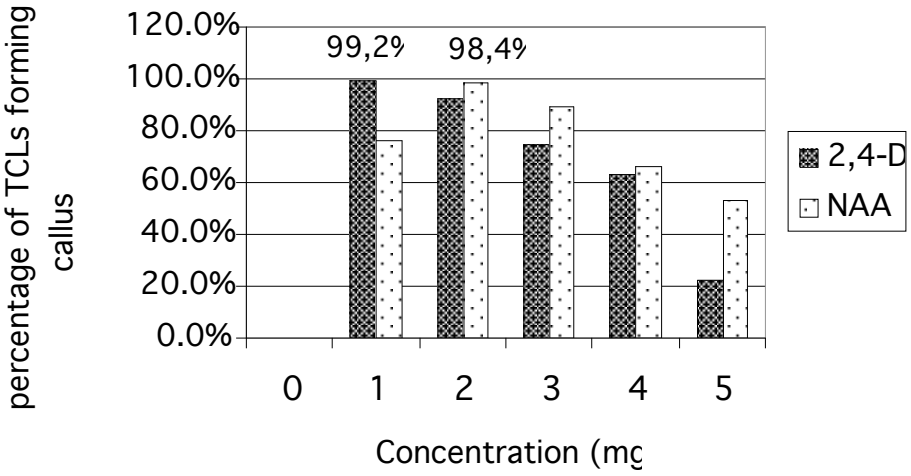
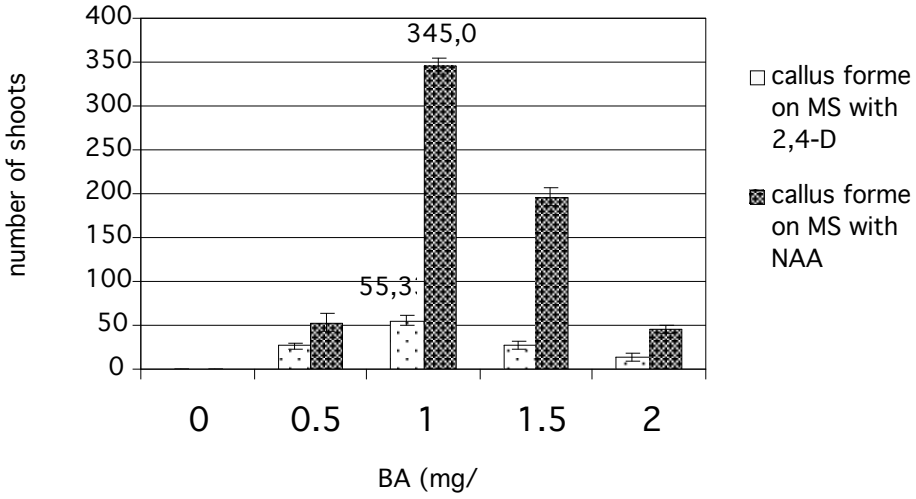


Fig 2. Effect of BA concentration on the number of shoots per callus



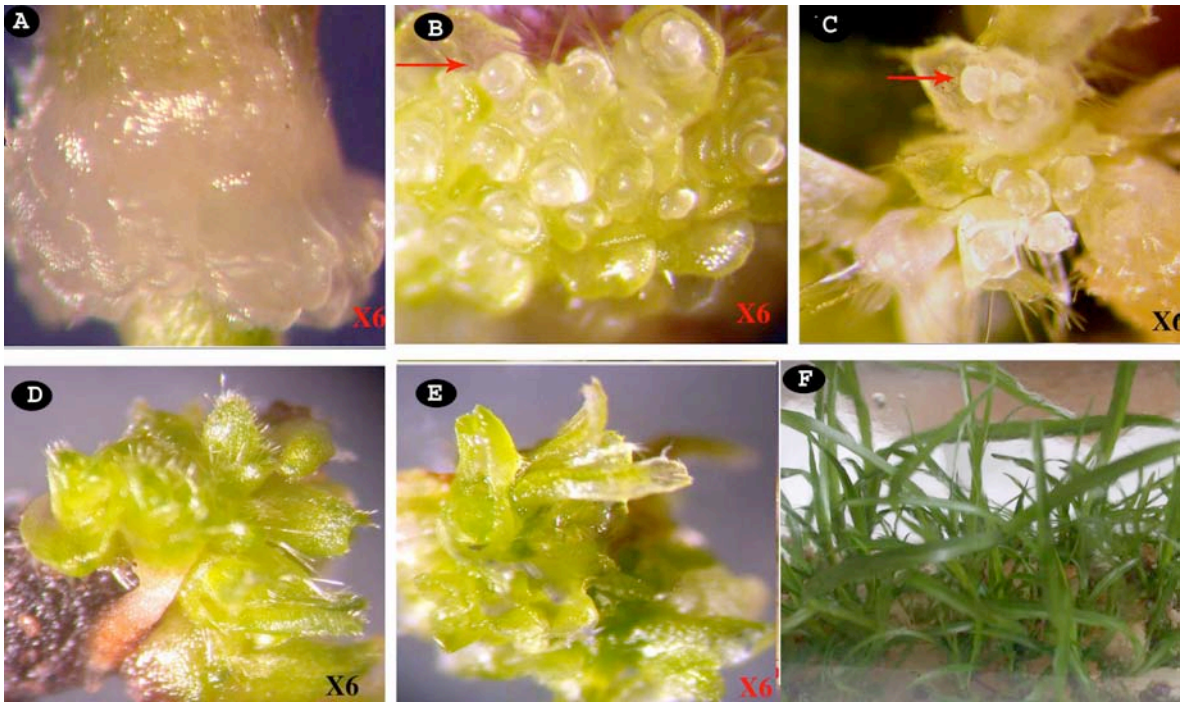
When the medium containing both 2 mg L^{-1} NAA and 1 mg L^{-1} BA , somatic embryos were formed from 97.53% of tTCLs of the young inflorescenes after two weeks. In the basic medium (MS) large number plantlets could be obtained by the somatic embryogenesis in *V. zizanioides* (tab.1) . The development of Vetiver somatic embryos (Fig. 3) was similar to the somatic embryo development of other monocotyledon such as *Digitaria sanguinalis (L.) Scop* (Bui Van Le, 1997) .

All of the *in vitro* plantlets survive in the nursery and even in the field.

Tab 1. Effect of ratio NAA and BA on the percentage of TCLs forming somatic embryos

NAA (mg/l)	BA (mg/l)	% TCLs forming somatic embryos
1	0.5	41.96
2	0.5	32.92
3	0.5	33.74
4	0.5	13.19
5	0.5	7.81
1	1	42.80
2	**1**	**97.53**
3	1	35.39
4	1	18.93
5	1	4.12
1	1.5	14.41
2	1.5	45.68
3	1.5	23.44
4	1.5	5.78
5	1.5	0.00
1	2	11.52
2	2	32.92
3	2	20.59
4	2	0.00
5	2	0.00

Fig 3. The development of somatic embryo formed TCLs



A: after 1 week; B: after 14 days; C: after 17 days; D: after 21 days; E: after 30 days; plantlets from somatic embryos

Selecting the salt tolerant callus clones

After subcultured five times, there were 8,89% of calli surviving on the medium containing 2.5% NaCl which formed the bud clusters when plated on the medium supplemented with 1 mg L^{-1} BA and 2,5% NaCl. The bud clusters developed into plantlet and grew well on the MS medium containing 2.5% NaCl whereas 100% unselectd plantlets (in control experiment) died when the medium was supplemented 1.5% NaCl after 2 weeks.

Tab 2. Percentage of surviving calli and the number of shoots after selection

Time of subculture and grow regulators	[NaCl] (%)	% surviving calli	Number of shoots per callus
1 (NAA 2 mgL^{-1})	0.5	96.67%	-
2 (NAA 2 mgL^{-1})	1	77.78%	-
3 (NAA 2 mgL^{-1})	1.5	44.44%	-
4 (NAA 2 mgL^{-1}l)	2	28.89%	-
5 (NAA 2 mgL^{-1})	2.5	8.89%	-
6 (BA 1 mgL^{-1})	2.5	8.89%	8.39±1.05

In the nursery all of the selected plantlets thrived normally on the soil supplemented 2.5 % NaCl on which unselectd plantlets (in control experiment) died completely.

In conclusion, a large number of Vetiver plantlets simply produced via embryogenesis and organogenesis form tTCLs of inflorescenes in a short interval not only satisfy the great demand of these for preserving soil now but also are an important potential of somaclonal variation for selecting excellent Vetiver varieties. In addition, embryogenesis is new and interesting for further research in vetiver grass as a model of other grass or cereal species. Finally, the Vetiver plantlets produced from selecting salt tolerant callus clones primarily apdapt to the saline soil in Vietnam.

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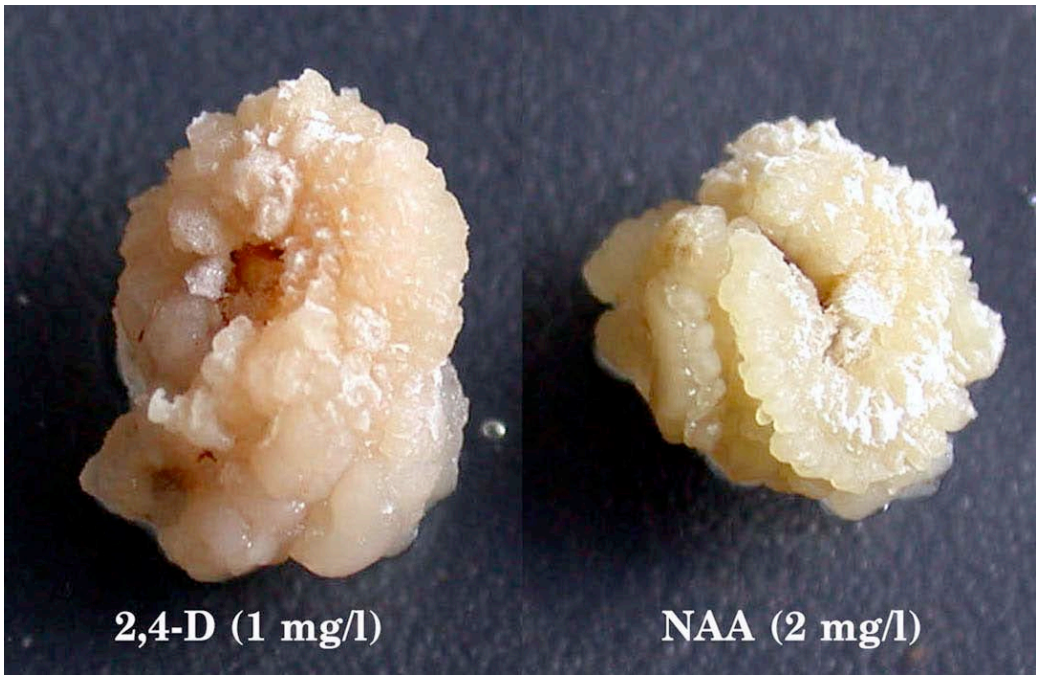
ADDITIONAL IMAGES:


B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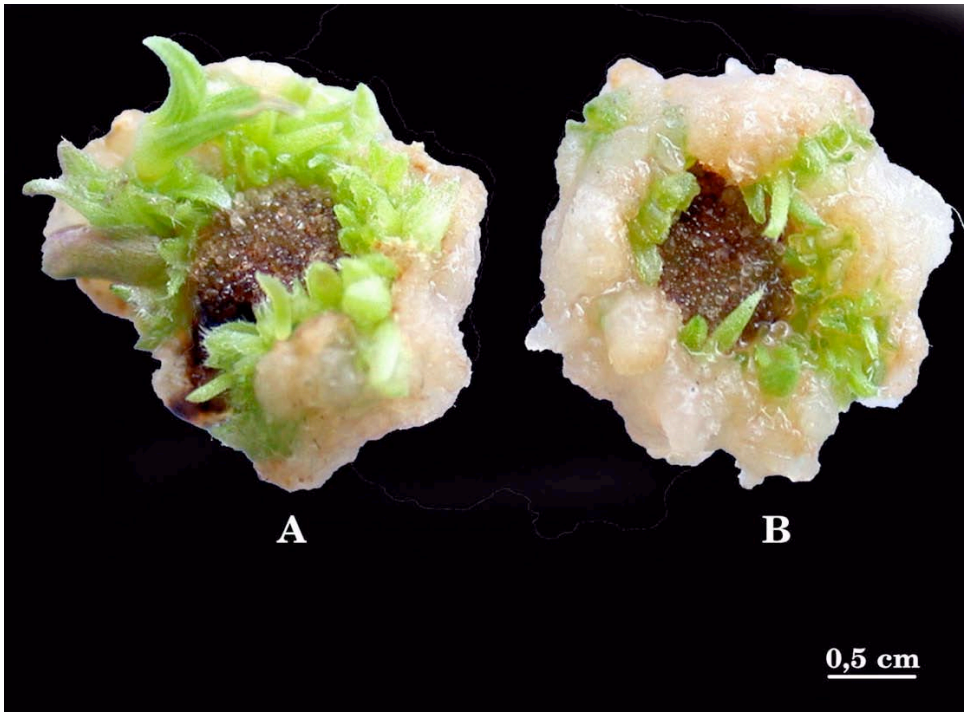
NAA (2 mg/l)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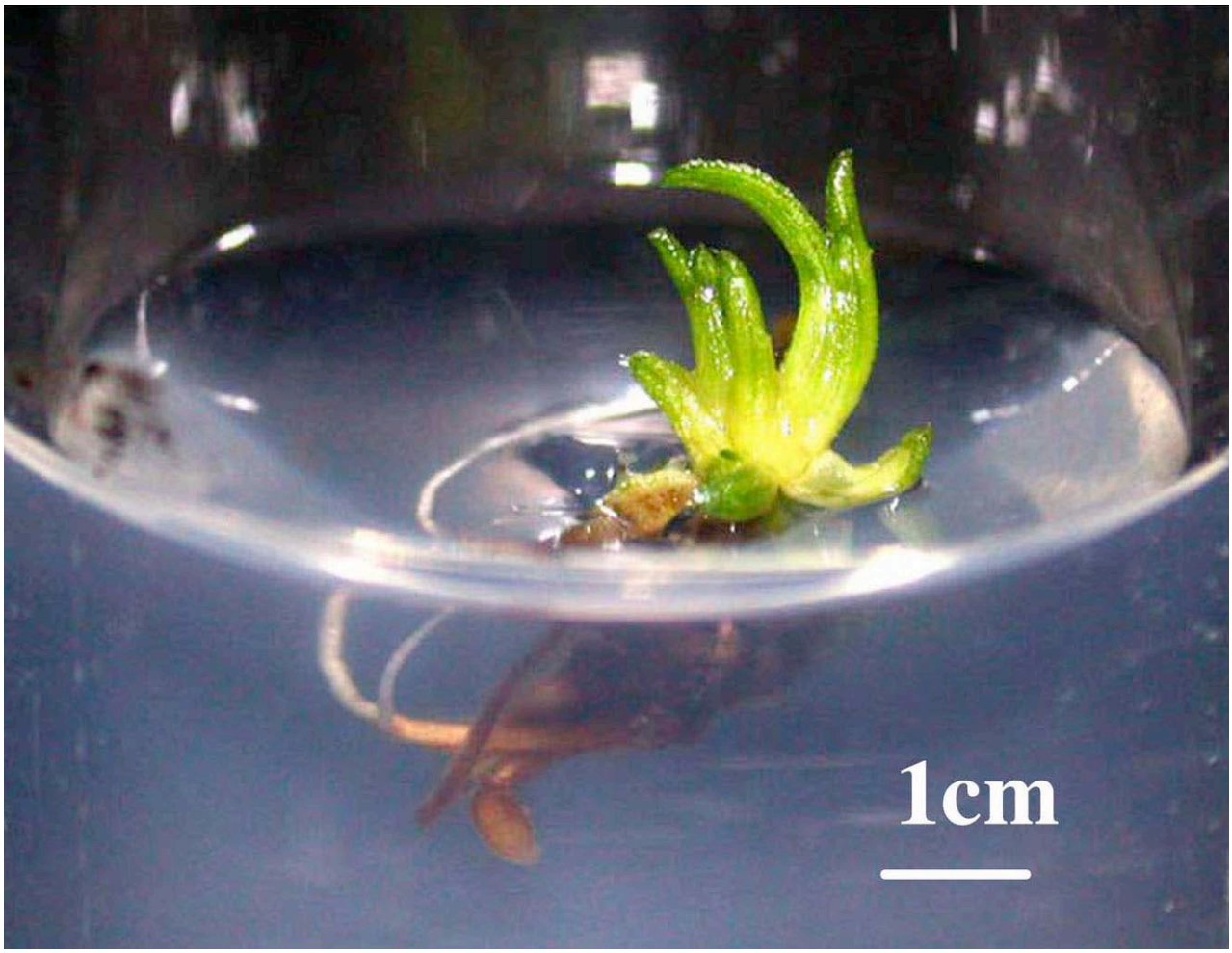
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