Interim Report on Breeding the New Vetiver Variety for Cold Tolerance by Biotechnology

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Culture *in vitro* is one basic research means of genetic transformation, and is also the technology precondition of modifying any plant's species quality; somatic embryogenesis is one of the ways of plant regeneration from culture in vitro, and is also the effective approach to improve regeneration frequency. In order to improve genetic quality of vetiver, especially in the aspect of cold tolerance, we carried out the research on somatic embryogenesis of vetiver and its cytology, especially its cytology characters and formation conditions of somatic embryogenesis. In addition, we also investigated the factors of somatic embryogenesis and plant regeneration of vetiver

1 Materials and Methods

1. 1 Experimental materials

The experimental materials contained 5 varieties, *Kandy, Karnataka, Malaysia, Sunshine*, and *Zomba*; they are native to Sri Lanka, India, Malaysia, USA, and Malawi, respectively, and regarded as good ones in the locality. The five ecotypes were first introduced into an experimental nursery in the US by Mr. Mark Dafforn and Dr. Robert P. Adams for a period of time, and then sent to us in July 1999. Afterwards, they have grown in the nursery of South China Botanic Garden. During the past years, *Karnataka* was found to have the fastest tiller forming speed and the shortest plant height, while *Zomba* assumed the slowest tiller forming speed in the nursery (This result was reported in the *Vetiverim* #22, P9-14).

1.2 Explant

Two kinds of explants were used in this experiment for culture *in vitro* of vetiver. One was nodes with axillary buds of the plant sampled from the field and the other was aseptic adventitious buds from organogenesis of cultured materials in test tube. When the former was used, cut the stalk with nodes and shucked off its sheath, then intercepted nodes with axillary buds and disinfected their surface using 70% ethanol, 20% hypochlorous natrium and 0.1% chloridize mercury in turn. Finally, inoculated the explants into the mediums. When the latter was used, took out adventitious buds from test tube under axenic condition and then inoculated as the former.

1. 3 Culture medium

The basal culture medium was MS medium (Murashige and Skoog Medium). It was supplemented with 6BA (6-Benzylaminopurine) and IBA (indole-3-butyric acid) when inducing organogenesis; supplemented with 2,4-D (2,4-Dichlorophenoxyacetic acid) and different confecting proportions of other growth regulators (such as auxin and cytokinin) when subculturing and inducing embryogenic calli; and supplemented with KT (Kinetin), 6BA and NAA (naphthalene acetic acid) when inducing plants regeneration.

1. 4 Differentiation, transplanting and planting of regeneration plants

Embryonic calli were transferred into the differentiation medium and then illuminated for 12 h/d (1200 lx); the incubation temperature was kept 25±2°C. After the regeneration plants developed from embryonic calli, they were transferred into the rooting medium for about 2 weeks, and then potted. Plantlets were planted on dry land when they were some 20 cm high. Kept them wet by irrigation and observed their growth performance after they became green.

2 Results That Have Been Gained

2. 1 Set up effective regeneration system of vetiver

Through the study on culture *in vitro* of vetiver axillary buds, a perfect vetiver somatic embryogenesis and plant regeneration technology was set up. The results showed that MS + 2,4-D (2.0 mg/L) + KT (0.5 mg/L) is the proper inducing medium and subculture medium; MS + 6BA (1.0 mg/L) is the proper differentiation medium; and 1/2MS + IBA (0.1 mg/L) + MET (Poclobutrazol) (0.1 mg/L) is the proper rooting medium. Up to now, we have obtained more than 10,000 cloning plants of vetiver, and also found out some mutants among them; the longest subculture time of embryogenic calli have reached 19 generations. Furthermore, we have possessed a batch of newly-induced embryogenic calli (1 generation). The establishment of vetiver regeneration system provides a staunch basis for its genetic transformation.

2. 2 Explored the factors of somatic embryogenesis and plant regeneration of vetiver

On the basis of establishment of the vetiver regeneration system, a series of experiments have been conducted to study the factors of somatic embryogenesis and plant regeneration of vetiver.

2. 2. 1 Somatic embryos inducing condition

Six kinds of inducing medium containing different confecting proportions of auxin and cytokinin were designed to induce somatic embryos of two culture materials (non-embryogenic calli and aseptic adventitious buds). It can be seen from Table 1 that, when there was no 2,4-D added into the medium, there were no somatic embryos occurred at the medium no matter what concentration 6BA was, 0.5 or 1.0 mg/L. On the contrary, when there was 2,4-D added, there were always somatic embryos occurred no matter whether or not 6BA was added. Moreover, the medium without 6BA had the highest inducing frequency (96.7%) for somatic embryos. When the content of 2,4-D reached 4.0 mg/L, about 62.2% aseptic adventitious buds could not be induced to produce somatic embryos. The most perfect confecting proportion of 2,4-D and 6BA for the aseptic adventitious buds to be induced was 1.0 : 0.5, and the inducing frequency was up to 79.5%. The quality of most somatic embryos was very good.

Integrating vetiver's reaction to growth hormone (2,4-D) and cell division hormone (6BA) in the medium when it was in culture *in vitro*, it could be inferred that explants (axillary buds) would form regeneration plants via organogenesis (adventitious buds) when the medium did not contain growth hormone but cell division hormone; on the contrary, explants would form regeneration plants via somatic embryogenesis when the medium contains only 2,4-D without or only a little 6BA. It indicates that 2,4-D is really the important factor for graminaceous plants to induce somatic embryogenesis when culture *in vitro.*

Table 1 Effect of different confecting proportions of auxin and cytokinin on inducing somatic embryos in vetiver

Culturing material*		Code of medium	2,4-D (mg/L)	6BA (mg/L)	Status of somatic embryos
Kinds	Amount (P)				
C	90	8	0	0.5	No E-callus
B	72	8	0	0.5	Only buds and leaves developed, no callus
C	93	9	0	1.0	No E-callus
B	64	9	0	1.0	Only buds and leaves developed
C	90	10	0.5	0	E-calli developed very well, only 3.3% pieces of calli were not E-callis
B	70	10	0.5	0	Only 10% buds developed into calli, but none was E-callus
C	97	11	1.0	0.5	E-callus developed well, and 14.1% pieces of calli were without E-calli
B	77	11	1.0	0.5	21.5% aseptic adventitious buds had no E-calli, and the majority of the others developed into very good E-calli
C	90	12	2.0	1.0	E-calli developed well, only 9.9% pieces of calli had no E-calli
B	78	12	2.0	1.0	61.5% of buds had no E-calli
C	92	13	4.0	2.0	E-calli developed well, 15.4% pieces of calli had no E-calli
B	90	13	4.0	2.0	62.2% of buds had no E-calli

* P = piece; C = non-embryogenic callus; B = aseptic adventitious bud; E-calli = embryogenic calli

2. 2. 2 Difference on inducing frequency of embryonic calli of different varieties

The results (Table 2) showed that there were prominent differences among the inducing frequency of embryonic calli of different vetiver varieties_among which *Zomba* had the highest inducing frequency, up to 96.7%, followed by *Karnataka*, 93.1%, while Malaysia assumed the lowest frequency, less than 30%, but all the varieties could be induced out calli at different levels (Fig.1: 1~5). This result offers causes to conduct experiment using different varieties purposefully.

Table 2 Differentia of inducing frequency of calli among diversified vetiver varieties

No.	Variety	No. of Explant	No. of calli (piece)	Frequency of calli induction (%)
1	*Kandy*	117	84	71.7
2	*Karnataka*	188	175	93.1
3	*Malaysia*	102	28	27.4
4	*Sunshine*	100	69	69.0
5	*Zomba*	237	229	96.7

* (1) Same medium for all varieties; (2) Data were from two replicates and investigation was conducted 15 d after inoculation.

2. 2. 3 Effects of low temperature condition on plant regeneration ability of embryonic calli

In order to breed and screen out cold-resistant germplasm, three groups of embryonic calli with different generations, all coming from *Karnataka*, were put into incubators with 3~7_, and then incubated with sunshine of 12 h/d (1200 lx) through different low temperature times, 98 days, 82 days and 82 days, respectively (Table 3).

Table 3 Effects of low temperature treatment (3-7_) on differentiation frequency of embryogenic calli in vetiver *Karnataka*

Generation	Duration (day)	No. of calli (piece)	Status of differentiation		
			No. of differentiation calli	Differentiation frequency (%)	Regenerated plantlets (cluster)
6th	98	84	34	40.5	78
13th	82	98	49	50.0	90
16th	82	280	135	48.2	235

The data in Table 3 indicated that parts of embryogenic calli would be no longer differentiated or even died, but some 40.5~50.0% of them still kept regeneration power under the 3~7°C low temperature condition (Fig.1: 6). Farther test would be done to confirm whether or not these embryogenic calli stressed by low temperature have the stronger ability to tolerate cold.

2.2.4 Plant regeneration of somatic embryos and their regeneration ability that may keep long

After embryogenic calli were transferred to the differentiation medium, they began to become green and gradually germinate in two weeks, and then developed into intact little plantlets. The embryonic structure and its regeneration ability can be kept long under the subculture condition. The pictures (Fig.1: 7~9) show that the growth status of regeneration plants grew quite well from the non-subculture (0 generation) to the 15th generation, which is impossible for other gramineous plants, such as rice. Table 4 shows that embryogenic calli subcultured for two years still had a very strong regeneration ability.

Table 4 Effect of subculture duration on regeneration frequency of embryogenic calli in vetiver *Randy**

Subculture duration (month)	No. of E-calli (piece)	Regenerated plantlets (cluster)	Regeneration frequency (%)
18	400	368	92.00
20	750	695	92.67
22	350	300	85.71
24	750	612	81.60

* (1) Data are from count every two months in recent six months; (2) Clustered plantlets regenerated from same callus are calculated as 1 cluster.

2. 3 Preliminary observation to the somaclonal variation

So far, many variations have been found among the over 10,000 clonal plants, including plant type (from erect type to creeping one), plant height (only dozens of centimeter high during the middle seedling phase), leaf color (from green to purple), leaf shape (from lance-shape to curled-shape), etc. (Fig.1: 10~11). All these differentiations above-mentioned

belonged to the profitable ones_farther research will be done to ascertain if these variations come from gene mutation and what kind of gene mutation it is.

2. 4 Cytology research of somatic embryogenesis and paper publication

In order to ascertain the cytoarchitecture of somatic embryos, they were taken out from the medium and then dipped in the fixation liquid containing carbinol-ice acetic acid (3:1). Thereafter they were sliced up according to general methods of olefin slice, whose thickness was 8_m, and then stained with Ehrich-hematoxylin. After mounting, they were observed and taken pictures under microscope to systematically observe the cytological feature of somatic embryogenesis (Fig. 2).

One paper has been published by the periodical *Acta Ecologica Sinica* (2003, 23(7): 1290-1296) by now, whose title is “Cytology observation and formation conditions of somatic embryogenesis in *Vetiveria zizanioides”*.

2.5 Allopatric planting of the somaclonal variations

We have established a favorable cooperation fellowship with Changzhou Biological Produces Co. Ltd. in Hebei Province, North China. A group of tube-seedlings were planted at its nursery in last August. And their growth status and process have been recorded and observed carefully. Soon, we are going there again to make a detailed investigation after the northern winter is over.

3 Further Working Plan

3.1 Continue to establish and preserve the regeneration system of embryonic calli of vetiver.

3.2 Continue to observe and record the existing materials, especially the mutants, and set up an information data bank.

3.3 Chemical induction: investigate the treatment effects of different concentrations of EMS on lethality rate, regeneration frequency and mutation frequency of embryogenic calli, etc.

3.4 Establish genetic transformation technology applying to vetiver: extrinsic genes (trehalose-6-phosphate synthase) are introduced into vetiver by *Agrobacterium*-mediated; gene gun is also considered to be used simultaneously.

3.5 Transplant and plant the regeneration seedlings that have been treated and transformed genetically in the nursery of South China Botanic Garden and in a cold region (Changzhou, Hebei Province) simultaneously, and then observe their growth habits and record them.

3.6 Set up a demonstration project using the newly-breeding vetiver variety that has a stronger ability to resist cold.

4 Outlay on the Project

We have received RMB 80 thousand Yuan, a half from TVN and the other from SCIB, for the project. So far over 80 thousand Yuan has been costed in the following items.

Item	Expenditure (RMB, Thousand Yuan)	Reason for expenditure

Purchase materials, reagents, medicals, etc.	23	Purchased lots of consumptive materials such as reagents, medicals, and tools, and utilization of water, electricity, glasshouse, etc.
Purchase equipments	17	Purchased a vibrator, an air-conditioner, and a computer for the project.
Use equipments	10	Utilization, maintenance and repair of pertinent equipments during the period of experiment.
Salary	22	About 12 thousand Yuan to a casual laborer for his annual salary, and the other 10 thousand Yuan to researchers for their subsidy.
Work trip	5	Last August, 2 researchers went to Changzhou, Hebei Province nearby Beijing to set up an experimental site and plant vetiver.
Others	6	Expenditure for paper publication, consumptive stationary, photography, etc.
Total	83	

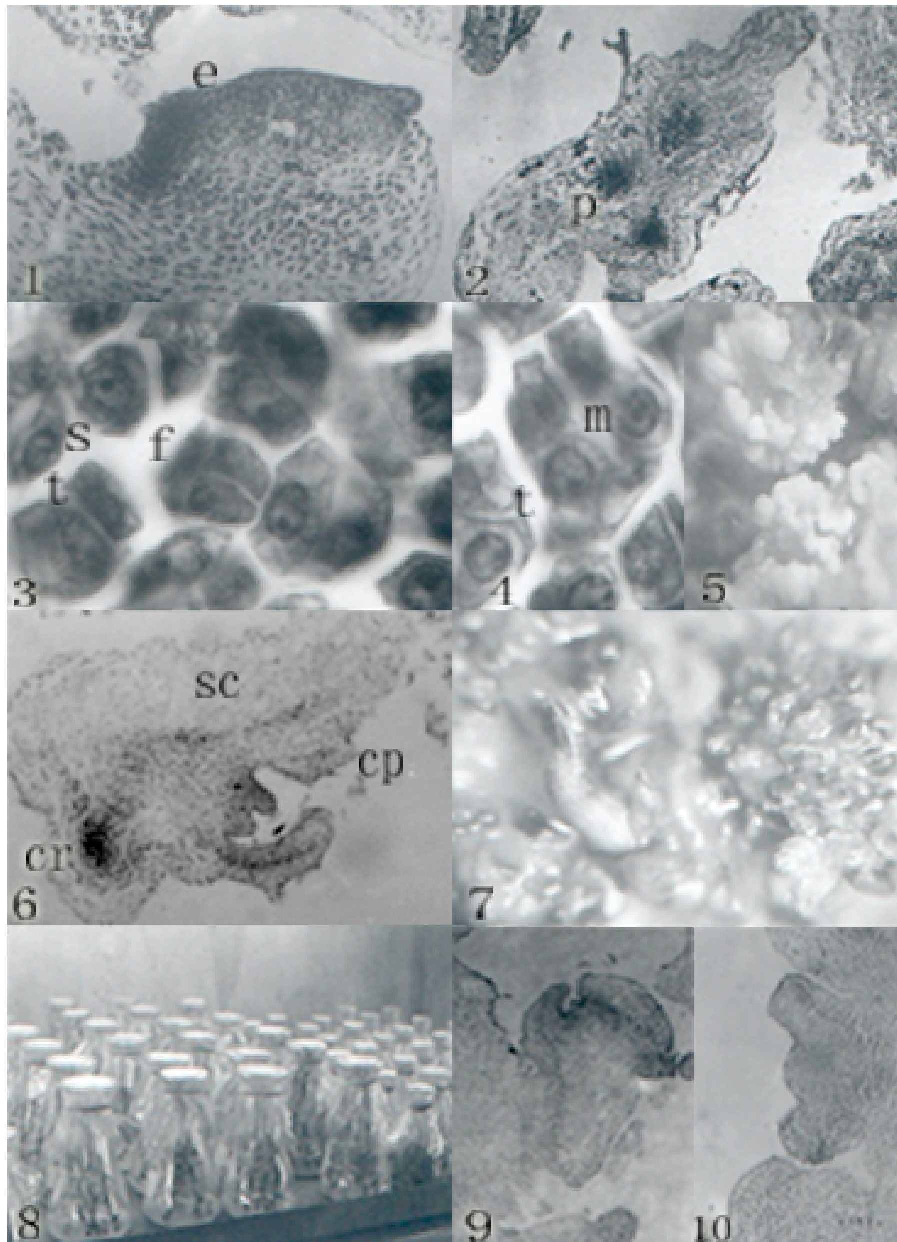

Fig. 1 Plants regeneration and variation plants of vetiver

1~5. Induction for somatic embryogenic calli of vetiver. They showed that E-calli were induced from 2#, 3#, 5#, 7# and 10#;

6. Effect of low temperature treatment on plant regeneration of embryogenic calli of vetiver. It showed that some embryogenic calli still kept differentiation ability under the 3~7 °C condition;

7~9. Embryogenic calli subcultured for long-term still had very strong regeneration ability. They showed that the growth status of regeneration plants were quite good all long from non-subculture (0 generation) to the 15th generation;

10~11. Variation plants from somatic clone. They showed many variation types, including plant type (from erect type to creeping type), plant height (only several decades cm during middle seedling time), leaf color (from viridescence to purple), leaf shape (from lance-shape to curled-shape), etc.

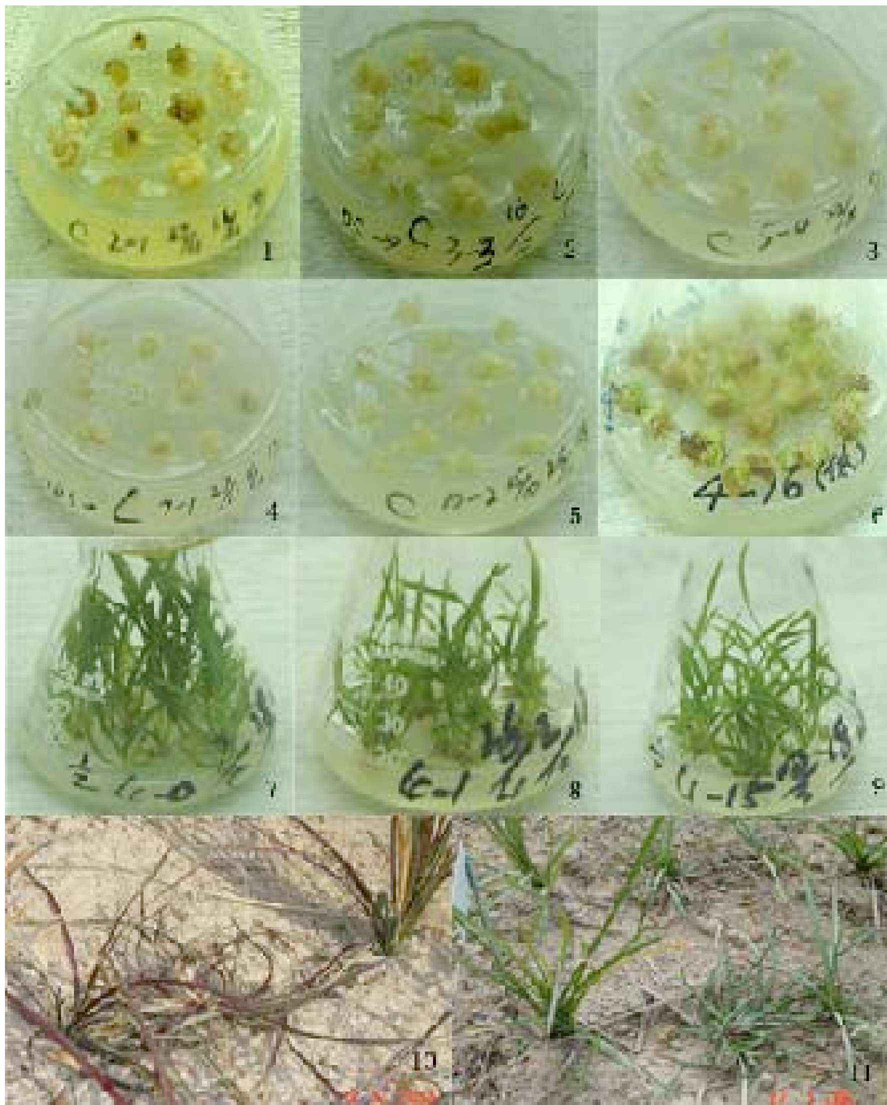

Fig. 2 Formation of somatic embryos and regeneration plants of vetiver

1. Epidermal cells (e) of the explant began to initiate division after two weeks of inoculation; _100
2. Vascular bundle and parenchyma cells (p) began to division after two weeks of inoculation; _100
3. Somatic embryo with proembryo at different stages: single cell (s), two cells (t), four cells (f); _1 000
4. Somatic embryo with multiple cells of proembryo (m); _1 000
5. Visible embryonic callus; _30
6. Single somatic embryos consisted of coleorhiza (cr), coleoptile (cp) and scutellum (sc), vascular bundles joined between the coleorhiza and coleoptile; _100
7. Embryonic cellus is in the differentiation period, each somatic embryo is budding; _100
8. A large number of regeneration plants from somatic embryogenesis;

9~10. Abnormal somatic embryo with torpedo proembryo which only exists in Dicotyledoneae. _100

Fig. 3 Tissue culture workshop

Fig. 4 The worker is conducting the experiment