LOW COST MICROPROPAGATION OF VETIVER GRASS

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Abstract

As the Vetiver System has proved to be a very effective and low cost mean of wave and flood erosion in the Mekong Delta, Vietnam, the demand for planting materials has increased enormously in the last few years. To response to this demand, a low cost micropropagation procedure for vetiver was developed by the College of Agriculture & Applied Biology, University of Can Tho.

Very young shoots removed from an active culm were used instead of tissues from other plant parts, and a liquid Murashige and Skoog medium supplemented with 2-4 mgBAl^{-1} gave the best multiplication medium, with an average of 8 new shoots during an incubation period of 6 weeks. Multiplication and rooting stage *in vitro* can be carried out under the natural conditions of a nethouse instead of growth chamber.

No statistical differences were observed between the two environmental conditions for propagation, rooting, and survival cluster after ten weeks of acclimatization. Plantlets produced under nethouse conditions were about 22% cheaper than growth chamber plants.

Keywords: Micropropagation, culm shoot, nethouse.

1.0 INTRODUCTION

In vivo propagation of vetiver is easy and simple, but its multiplication rate is low, and requires a lot of initial planting materials. *In vitro* propagation has many advantages such as: high rate of multiplication, smaller land area and production of uniform shoots. However, the micropropagated shoots are relatively expensive because production cost includes the expensive growth chamber.

To reduce costs of micropropagation, scientists have tried to develop other procedures, for instance: automatic subculturing (Hartney, 1986), using natural daylight to promote photoautotrophic growth (George, 1993), large scale production using a temporary immersion system (Escalona *et al*., 1998), periodic immersion bioreactor (Firoozabady and Gutterson, 2003), an alternative for artificial light (Kodym and Zapata-Arias, 1999; 2001), tubular skylight (Kodym *et al*., 2001), the natural-light of a nethouse (Be and Debergh, 2006).
The proliferation stage is the longest stage because it requires several steps for subculture. The rooted period also needs time, long enough for stem elongation and establishment of a new root before acclimation stage. So the demand for electricity for light and temperature control in the growth chamber is huge (24±1^{o}C under a 12-h photoperiod provided by fluorescent tubes). So if two of these stages can be carried out under nethouse conditions (natural light and temperature), large saving on energy will lead to the production of low cost plantlets.

Therefore the aim of these experiments was to lower the price of micropropagated plantlets by carrying out stage II (proliferation) and III (*in vitro* rooting) under natural conditions of a nethouse.

2.0 MATERIALS AND METHODS

2.1. Shoot Induction (Stage I)

A flowering culm was used, and the nodes at 1.2 m high in the culm were chosen to avoid infections. The 20cm segments with lateral buds were cultured into glass tubes containing Murashige and Skoog's medium (1962) [MS] after sterilization. This stage was carried out under growth chamber conditions (24±1^{o}C, 12-h photoperiod provided by fluorescent tubes with a photosynthetic active radiation (PAR) of about 30 µmol.m^{-2}.s^{-1}.

2.2. Proliferation Stage (Stage II)

Materials of proliferation were the young shoots collected from Stage I, with 5-6 leaves and about 4 cm high,. The young shoots were subcultured in jars of the same MS medium with different hormone supplements: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mgBAl^{-1} (benzyl adenine). The experiment was designed as a randomized complete block with 4 replicates, 5 jars per replicate, 2 single shoots per jar. The jars were glass (6 cm diameter, 12 cm high), closed with a plastic cap and wrapped in polyvinylchloride film before placing in the nethouse conditions (30±1^{o}C at 11:00 and 31±1^{o}C at 15:00, under 8-10 daylight with a PAR varying between about 95-125 µmol.m^{-2}.s^{-1}) and under growth chamber conditions (24±1^{o}C under a 12-h photoperiod provided by fluorescent tubes with PAR of about 30 µmol.m^{-2}.s^{-1}). Twenty mL of liquid MS medium were used per container. As the young shoots were not submerged in this amount of liquid medium, the jars were kept stationary without shaking.

Data were analyzed by a two-way analysis of variance, in which shoot number, clump weight, shoot height, chlorophyll a, b content after 6 weeks in culture were the dependent variables. The independent variables were the physical environments for multiplication (growth chamber or nethouse) and 10 concentration of BA (mgl^{-1}). The content of pigments was analyzed according to Wellburn (1994).

2.3. In Vitro Root Initiation (Stage III)

Clump from Stage II contained average 7-9 shoots, it was subdivided into smaller clump (4-5 shoots per clump), those with shoots longer than 4 cm, were used for root initiation. The liquid MS medium were supplemented with two levels of sucrose (3% and 4%), and modified NAA (naphthalene acetic acid) with 0 and 1 mgl^{-1}. The plastic containers (12 cm diameter, 7 cm high) contained 10 clumps (4-5 shoots per clump). Data were analyzed by a three-way analysis of variance in which root number/clump, weight of clump, height of plantlets were the dependent variables; the independent variables were the environmental conditions (see proliferation stage), two concentrations of NAA (0 and 1 mgl^{-1}) and sucrose (3% and 4%) for root initiation.

2.4. Acclimatization (Stage IV)

Clump of shoots from Stage III was acclimatizated in the nursery in trays containing soil with high organic content. These clumps were kept in 130 - 140 $\mu molm^{-2}$ s^{-1}, 70-80% relative humidity of air. Water was spayed manually 4 times per days during the first week of acclimatization. The survival ratio of clump was observed 8 weeks after acclimatization in the nursery. The experiment was carried out completely randomized design with 5 replicates, 100 clumps per observation.

3.0 RESULTS

3.1. Shoot induction (Stage I)

Nodes with lateral buds were cultured into the MS medium without hormones. The shoots appeared 14 days after inoculation (Photo 1 and Photo 2). There were only 14% of lateral buds which appeared a new shoots, the rest (ca. 86%) were contaminated by micro-organisms. The size of the lateral buds determined the contamination level: the larger size buds had more infection. The size of the buds is most important; successful rate of 1 mm buds is 74% and decreased to 21% in 3mm buds (Enjalric *et al*., 1988).

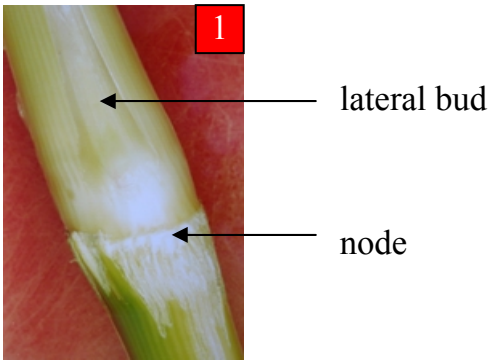

Photo 1: A culm with lateral bud was used

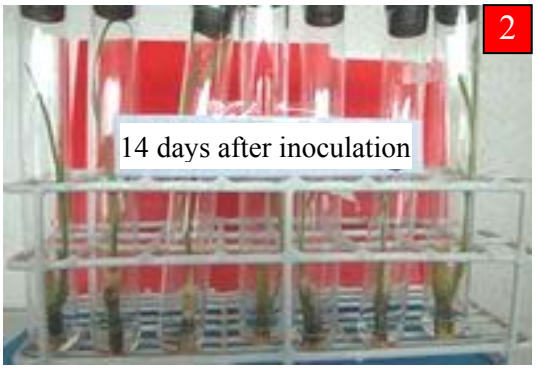

Photo 2: The new shoots appeared 14 days after inoculation into MS medium

As shown in Table 1, the number of useful new shoot (more than 4 cm high) varied with BA concentration in MS medium. The results can be divided into 4 groups: *(1)* 0 ($mgBAl^{-1}$); *(2)* 1 ($mgBAl^{-1}$); *(3)* 2-4 ($mgBAl^{-1}$) and *(4)* over 4($mgBAl^{-1}$). Generally, concentrations of BA from 2-4 mgl^{-1} were best for vetiver proliferation; average with 8 shoots after 6 weeks culture, with 4-5 cm high shoots. Higher concentration of BA were not effective, they stimulated too many small shoots (0.8-2 cm high), which can not be used either for rooting or subculture (Photo 4). Similarly, clump mass was also varied with on BA concentrations.

The physical environment during in vitro culture did not affect the observed parameters such as shoot number, shoot-cluster weight. Moreover, the chlorophyll a content was 142 $\mu g.g^{-1}$ for growth chamber and 168 $\mu g.g^{-1}$ for nethouse conditions, which did not statistically different ($P \leq 0.05$). The chlorophyll b content was the same chlorophyll a in the experiment.

In general, the results (Table 1) show that nethouse conditions did not have a negative effect on proliferation stage of vetiver. Therefore environmental conditions of nethouse can be used instead of the growth chamber for multiplication to save costs.

Photo 3: Vetiver was proliferated in the glass jars which contain MS medium supplemented 2 mgBAl^{-1}, 6 weeks after culture.

Photo 4: Vetiver proliferation in the medium supplemented 10 mgBAl^{-1}, 6 weeks after culture

Table 1: Effect of hormonal treatment [(1)] (HT) and environmental conditions [(2)] (EC) during in vitro culture on the development of auxiliary shoots after 6 weeks

Concentration of BA (mg/l)	Shoot number /clump [(3)]	Clump mass (g)	Average high shoots (cm)	Chlorophyll a (µg/g)	Chlorophyll b (µg/g)
0	1.0 d [(4)]	0.2 f	9.1 a	129.2 cd	130.6 bcd
1	6.3 b	1.0 abc	5.1 b	194.1 ab	182.0 a
2	8.1 a	1.1 a	5.0 b	170.4 abc	190.4 a
3	7.8 a	1.0 ab	4.9 b	170.4 abcd	155.5 abc
4	8.2 a	1.1 a	4.6 b	114.2 d	109.4 de
5	4.0 c	0.7 cd	2.3 cd	143.7 bcd	134.0 bcd
6	4.4 c	1.1 a	2.9 c	132.0 cd	112.9 de
7	1.1 d	0.8 bcd	1.7 de	149.5 bcd	119.9 cde
8	0.8 d	0.6 de	1.3 e	146.6 bcd	110.7 de
9	1.6 d	0.3 ef	1.3 e	221.6 a	163.2 ab
10	0.2 d	0.3 ef	0.8 e	167. 3 abcd	90.2 e
Environmental conditions					
Growth chamber	4.1	0.7	3.6	142	121
Nethouse	3.8	0.8	3.4	168	156
F test (P≤0.05)					
F test HT	*	*	*	*	*
F test EC	ns	ns	ns	ns	ns
F test HT vs EC	ns	ns	ns	ns	ns
CV (%)	32.9	32	22	23.4	20

[(1)] Different concentrations of BA from 0-10 mgBA/l

[(2)] Two environmental conditions: growth chamber (24±1^{o}C under a 12-h photoperiod provided by fluorescent tubes with PAR of about 30 µmol.m^{-2}.s^{-1}), and nethouse 30±1^{o}C under 8-10 daylight with a PAR varying between about 95-125 µmol.m^{-2}.s^{-1}.

[(3)] Shoot number/clump is only included the shoots which are longer 3 cm

[(4)] Values followed by the same letter are not significantly differently at P≤0.05 of Duncan's test. The significance of the calculated F values is: ns, not significant; *, significant P≤0.05.

3.3. In Vitro Root Initiation (Stage III)

Almost all new shoots produced on culture media with ($mgBAl^{-1}$) at 0, 1, 5, 6, 7, 8, 9, and 10 were rejected, because their height did not satisfy the root initiation requirements (either too short or too long). Only shoots approximately 4cm in height from treatments of 2 to $4mgBAl^{-1}$ were used. The morphological parameters of rooted plantlets which reached approximately 7 cm after 10 days in root initiation medium are presented in Table 2. Under both environmental conditions, root establishment was satisfactory; no statistical differences were observed in root number/clump, clump weight, and height of shoot. Adding 1 $mgNAAl^{-1}$ in the MS medium affected the root number per clump (average 19 roots/clump) and 7.6 roots/clump for 0 $mgNAAl^{-1}$. This difference was statistically significant by the F test ($P \leq 0.05$). Whereas, sucrose content had no effect on the observed parameters of plantlets except shoot height. Adding 40 gL^{-1} of sucrose in medium inhibited the height of shoot because the high content of sugar (40 gL^{-1}) enhanced the new shoots as shown in Table 2.

Table 2: Effect of NAA, environmental conditions (EC), and sucrose content on root initiation in vitro of clumps (4-5 shoots/clump) after 10 days in root initiation medium

Categories	Root number/clump	Clump weight (g)	Height of shoot (cm)
Treatments			
0 mg NAA + 30 g sucrose/liter	7.5 a	0.34	7.3 b
1 mg NAA + 30 g sucrose/liter	19.6 b	0.39	6.9 ab
0 mg NAA + 40 g sucrose/liter	7.6 a	0.35	6.7 a
1 mg NAA + 40 g sucrose/liter	18.5 b	0.34	6.4 a
F (treatments)	*	Ns	*
Environmental conditions (EC)			
Growth chamber	12.7	0.35	6.9
Nethouse	13.8	0.36	6.8
F (EC)	ns	Ns	ns
Concentrations of NAA (NAA)			
0 mg/liter	7.6	0.34	7.0
1 mg/liter	19.0	0.36	6.7
F (NAA)	*	Ns	*
Concentration of sucrose (Sucrose)			
30 g/liter	13.5	0.37	7.1
40 g/liter	13.0	0.34	6.7
F (sucrose)	ns	Ns	*
F (EC vs NAA vs Sucrose)	ns	Ns	ns
CV (%)	13.5	64	5.6

Photo 5 and 6 show the roots on shoot after 10 days in culture

Photo 5: Clump of vetiver developed roots in the plastic container which contained MS medium

Photo 6: Clump of vetiver with roots after 10 days in culture

3.4. Acclimatization (Stage IV)

The survival ratio is the most important parameter which decides the effectiveness of a micro-propagated protocol. There are many factors which affect the survival of clusters such as quality of plantlets and ex-vitro environments. The results show that their survival during the acclimatization was satisfactory (Table 3). The cluster of shoots survived with a high ratio (over 95%) and it developed well after 10 weeks weaning (Photo 7, 8, and 9). It can be concluded that the micro-propagated plantlets of vetiver well adapted under the natural conditions of nursery.

Table 3: Survival ratio of clump (4-5 shoots/clump) after 10 weeks after weaning in nursery

Treatment of pre-rooted *in vitro*	Survival ratio (%)
Growth chamber	
1 mg NAA + 40 g sucrose/liter	98,4
0 mg NAA + 30 g sucrose/liter	97,3
1 mg NAA + 30 g sucrose/liter	97,5
0 mg NAA + 40 g sucrose/liter	98,3
Nethouse	
0 mg NAA + 30 g sucrose/liter	96,3
1 mg NAA + 30 g sucrose/liter	98,8
0 mg NAA + 40 g sucrose/liter	99,7
1 mg NAA + 40 g sucrose/liter	99,6
F (treatment)	Ns
CV (%)	2,0



Photograph 7: The clump of Vetiver is acclimatized in the nursery
Photograph 8: The clump of Vetiver develops well after 8 weeks in the nursery
Photograph 9: The micro-propagated shoots can be used as materials for planting in the field.

4.0 DISCUSSION

The micro-propagation method of vetiver is better because it does not promote mutation; besides, vetiver plantlets are relatively small as compared to conventional tiller in polybags, make it easy for transporting large quantities to other areas (Namwongprom and Nanakorn 1992; Charanasri *et al.* 1996; Sukkasem and Chinnapan 1996). However, the tissue-culture plantlets are rarely used because of their cost production. But the environment of the nethouse is satisfactory so it can be employed for multiplication and root initiation *in vitro* to reduce costs.

Our results and calculations (data not presented in the text) show that the cost-price of micro-propagated plants under nethouse conditions are about 22% lower compared to growth chamber conditions. The lower cost was almost entirely due to the reduced energy costs for light and air conditioning. Debergh and Read (1991) reported that lighting costs account for 65% of the total electricity bill, and are one of the highest non-labor costs in a tissue culture laboratory (Dooley, 1991). Compared with other techniques, for example, the periodic immersion bioreactor (10L Nalgene vessels) for propagation *in vitro*, the cost price of 'Cayenne' pineapple propagules was decreased by 35% (Firoozabady and Gutterson, 2003). An earlier study showed that daylight instead of artificial light for banana propagation allowed savings on costs for electricity of US$6 m^{-2} $week^{-1}$, as compared to a standard growth room (controlled light intensity and temperature regimes) (Kodym *et al*., 2001), and 20% lower cost for pineapple micropropagation (Be and Debergh, 2006).

Micropropagated plants produced under outdoor conditions are not only cheaper, but the process is also environmentally friendly. Despite being economical Kyte (1987) did not recommend use of natural energy because of its fluctuations and difficulties to manage. However, southern Vietnam is located in a tropical region, with full sunlight over the whole

year and no large variations in temperature, so that natural light conditions can be used for micropropagation at lower cost. If the protocol of stages II and III were carried out under outdoor conditions, supplemented with 2 mgBAl^{-1} in the MS medium, it can be used and produced millions of micro-propagated plantlets each year for the urgent requirement of the Mekong Delta of Vietnam.

5.0 CONCLUSION

With a concentration of BA (2-4 mgl^{-1}) in liquid MS medium, it is possible to achieve a micro-propagation ratio of average 8 new shoots per single explant with a culture interval of 6 weeks. Shoots proliferating on these media have an approximate height of 4 cm. Lower or higher BA concentrations yielded shoots of lesser quality. Proliferation and root initiation stages in the micro-propagation of vetiver can easily be carried out under the natural ambience of a nethouse. The growth of plantlets produced under these conditions have a similar quality to those raised under growth chamber conditions. This protocol may reduce costs by up to 22%.

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A Brief Introduction to the First Author

Dr Le Van Be is the Head of the plant propagation unit of the University of Cantho, Vietnam. He is an active member of the Vietnam Vetiver Network and is responsible for producing planting materials for various VS applications in the Mekong Delta of southern Vietnam. In a the last 3 years he has developed a very effective and low cost method of micropropagation for vetiver to meet the increasing local demand for vetiver grass.