Photoautotrophic Tissue Culture of Cowpea (Vigna unguiculata L Walp.)

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Abstract: Developing a system for producing high quality transplants at low cost is important for resource saving, environmental conservation and biomass production. Photoautotrophic systems have reduced potentials of contamination, which facilitates success of large scale operations. Chlorophyllous explants in vitro have the ability to grow photoautotrophically. The regeneration frequency (80%) of cowpea (TVU-867-1B-2-7 and TVU-947-1997) embryo explant via photoautotrophic tissue culture was comparable ($p<0.005$) to the Photomixotrophic protocol. There was no significant varietal response in plant biometrics to both protocols.

This photoautotrophic protocol (Natural Ventilation System - NVS) ensured appropriate conditions for gaseous exchange in tissue culture vessel under natural ventilation and proffers an alternative to the improvement of plantlet physiology, operation and management in the mass propagation of cowpea at reduced production costs.

Keywords: Photoautotrophic, Photomixotrophic, Tissue Culture, In vitro, cowpea.

INTRODUCTION

Micropropagation, an *in vitro* vegetative method using pathogen-free propagules, has been considered significant in agriculture and forestry for producing pathogen-free stock plants or genetically superior clones that cannot be propagated by seeds. However, the widespread use of micropropagated transplant is still limited due to high production costs, low growth rate, low percentage survival at the *ex vitro* acclimatization stage and high labour costs. In a closed vessel, plantlets are unable to achieve their photosynthetic capacity as the carbon dioxide concentrations are too low during most of the light period. (Kozai, 1991). Increasing light levels and improving the availability of carbon dioxide, either through venting or artificial enrichment, allows plant tissue to be grown photoautotrophically in a vessel. Photoautotrophic micropropagation refers to micropropagation with no exogenous organic compound (sugar, vitamins etc) added to the growth medium. Development of photoautotrophic micropropagation for the production of

plantlets has shown that this method raises carbon dioxide and light levels and the lack of sucrose in the medium increases the growth of the plantlets (Zobayed, *et al*., 2000). The gaseous environment within the culture vessels can influence *in vitro* tissue growth since plant tissues generate and absorb gases, altering the gas composition. Poor gas permeability due to culture vessel closure gradually decreases CO2 concentrations thus explaining the low photosynthesis in vitro (Falque *et al*. 1991). Extensive studies have been carried out to demonstrate the relationship between the extent of vessels closure and the ability of plant tissues to take up carbon dioxide for photosynthesis in the absence of sucrose in the medium (Kozai, 1991). It was observed that plants can grow vigorously in the absence of sugar if provided with proper ventilation system coupled with high photosynthetic photon flux (PPF) of 100-150 µmol m -2 s -1 and lower percentage of relative humidity inside the culture vessel (Chun and Kozai 2001). This study was conducted to evaluate cowpea growth performance under photoautotrophic and photomixotrophic tissue culture conditions to develop a cost effective alternative to the relative complexity of the techniques and knowledge required for controlling the photoautotrophic *in vitro* environment, expense for lighting and CO2 enrichment.

MATERIALS AND METHODS

Source of plant material

Viable seeds (TVU-867-IB-2-7 and TVU-947-1997) of cowpea (*Vigna unguiculata)* were otained from seed gene bank of National Center for Genetic Resources and Biotechnology, Moor Plantation, Ibadan. Nigeria.

Seeds of cowpea were surface sterilized in for 5 min 70% ethanol and shaken for 5 min in a gyrator shaker at 100 rpm. The seeds were taken out, and introduced in 5% sodium hypochlorite for 20 min. To achieve double sterilization, the seeds were further placed in a 10% sodium

hypochlorite containing 2mls of tween 20 per 1000ml solution. The seeds were rinsed 3 times in sterile distilled water. The seeds were spilt opened with embryo attached to one cotyledon using sterile forceps and scalpel. The embryo was excised just at the nodal point. Decapitated embryo explants were placed horizontally on their respective nutrient medium for *in vitro* Photomixotrophic and Photoautotrophic culture. The experiment was conducted in a complete randomized block design and replicated twice with five explants per replicate.

According to procedure described by Murashige and Skoog (1962), basal media for photomixotropic culture was prepared using 30 g/l sucrose. The media was supplemented with 1.8ml of 0.1mg/l α- napthaleneacetic acid (NAA), 45mls of 0.1mg/l 6-benzyladeninepourine (BAP), 16.67ml/l of vitamin mixture (thiamine, niacin and pyridoxine) and 0.03g/l of inositol in a 300ml nutrient solution solidified with 7% agar. The agar was added after adjusting the pH of the media adjusted to 5.7 ± 0.1 with 0.1 N KOH or 0.1 N HCl before autoclaving for 20 min. The media were autoclaved at 121^{o}C for 15 minutes. All cultures were maintained in Plant Growth Chambers at 24±2°C at 16 h light photoperiod with cool white fluorescent light. Cultured test tubes (18 x 150mm) were covered and sealed with transparent polypropylene film of 0.2ul pore size. This system was adopted to improve gaseous exchange. Similar procedure of growth media preparation for Photomixotrophic systems was adopted for the photoautotrophic system; however, the growth medium was without sucrose and vitamins (Table 1).

The Photomixotrophic system was grown in a growth room and maintained at 26^{o}C under 16hr/8 hr (light/dark) photoperiod using white fluorescent tubes while the photoautotrophic system was placed in the screen house. The cultured plants were encased in a perforated transparent plastic film to keep the temperature under cover higher than the room temperature during the night and to avoid 100% humidity. The ventilator openings were controlled manually during hot (39^{oc}) days to decrease the room air temperature. The capping systems and growth environment was

considered for the C_{out} and C_{in} for the difussion of CO_2 with $C0_2$ –D-TH-RS (Temco controls). The photoautotrophic and photomixotrophic investigations lasted for 15 days.

Plantlet growth parameters assessed were for shoot length, root length and number of leaves. Parameters like chlorophyll and carbohydrate content were not reported due to technical malfunction. Data was subjected to analysis of variance (ANOVA) with mean separation (P = 0.05) by Duncan's Multiple Range test (DMRT).

Table 1: Photoautotrophic and Photomixotrophic Media Composition.

Reagents	Photoautotrophic(300mls)	Photomixotrophic (300mls)
Stock I	50ml	50ml
Stock II	5ml	5ml
EDTA	0.0746g	0.0746g
Fe	0.0556g	0.0556g
Vitamins mix.	0ml	5ml
Inositol	0.01g	0.01g
BAP	45mls 0f 0.1mg/ml	45mls of 0.1mg/ml
NAA	1.8ml of 0.1mg/ml	1.8ml of 0.1mg/ml
Agar	7g	7g
Sucrose	0g	30g

RESULTS

The oxygen, carbon dioxide and water vapour permeability through the polypropylene film in the growth house and glass house differed due to existing environmental conditions. Oxygen diffusion out of the polypropylene film in the growth room was lower than in the glass house. A similar result was obtained regarding the permeability of carbon dioxide as it was higher in the screen house due to influx of atmospheric carbon dioxide in the culture vessel. However, the water vapour escape from the culture vessel in the growth room was higher than obtained in the glass house (Table 2).

The analysis of variance indicated significant ($p<0.05$) differences among varieties subjected to photoautotrophic and Photomixotrophic systems (Table 3). The regeneration frequency (80%) of cowpea (TVU-867-1B-2-7 and TVU-947-1997) embryo explants via photoautotrophic tissue culture was comparable ($p<0.05$) to the photomixotropic system. Root regeneration and leaf outgrowth in the cowpea (TVU-867-1B-2-7 and TVU-947-1997) varieties were similar ($p<0.05$) However, shoot regeneration of TVU-867-1B-2-7 under photoautotrophic system was significantly ($p<0.05$) higher (3%) than in Photomixotrophic system.

Table 2. Ventilation Characteristics of the photoautotrophic and photomixotropic systems.

SCREENING ENVIRONMENT	OXYGEN PERMEABILITY (cm3/m2. 24 h. atm)	CARBONDIOXIDE PERMEABILITY (cm3/m2. 24 h. atm)	WATER VAPOUR PERMEABILITY (g/m2/day)
Growth Room	9,200	21,100	32.0
Glass House	13,100	30,200	12.5

Table 3: Root length (cm), Shoot length (cm) and Leaf number of the two cowpea cultivars under photoautotrophic and Photomixotrophic conditions, using embryo explants.

VARIETY 1: TVU-947-1997 (BROWN)	VARIETY 1: TVU-947-1997 (BROWN)
MS MEDIA WITH SUCROSE	MS MEDIA WITHOUT SUCROSE
EMBRYO CULTURE (Growth Room)	EMBRYO CULTURE (screen House)

PLANTLETS	ROOT LENGTH(cm)	SHOOT LENGTH(cm)	LEAF NUMBER	ROOT LENGTH(cm)	SHOOT LENGTH(cm)	LEAF NUMBER
1	1.9	3.1	2.0	2.0	3.4	2.0
2	2.1	2.2	2.0	2.0	2.7	2.0
3	1.7	4.5	3.0	1.5	3.9	3.0
4	1.5	4.9	3.0	1.6	4.2	3.0
5	2.0	3.5	2.0	1.8	3.7	2.0
6	2.1	3.3	2.0	2.1	3.5	2.0
7	1.5	3.2	2.0	2.1	2.9	2.0
8	1.8	3.6	2.0	1.9	3.6	2.0
9	1.5	3.9	2.0	1.4	4.2	2.0
10	1.5	3.4	2.0	1.6	3.8	2.0
MEAN±SE	1.76±0.09[a]	3.56±0.23[a]	2.20±0.10[a]	1.8±0.10[a]	3.59±0.29[a]	2.20±0.10[a]
	VARIETY 2: TVU-867-IB-2-7 (WHITE)			VARIETY 2: TVU-867-IB-2-7 (WHITE)		
	MS MEDIA WITH SUCROSE			MS MEDIA WITHOUT SUCROSE		
	EMBRYO CULTURE (Growth Room)			EMBRYO CULTURE (Screen House)		
PLANTLETS	ROOT LENGTH(cm)	SHOOT LENGTH(cm)	LEAF NUMBER	ROOT LENGTH(cm)	SHOOT LENGTH(cm)	LEAF NUMBER
1	1.9	1.6	2.0	1.2	1.7	2.0
2	1.0	1.2	2.0	1.7	1.8	2.0
3	1.3	1.5	2.0	1.5	1.5	2.0
4	1.2	1.4	2.0	1.2	1.5	2.0
5	1.1	1.3	2.0	1.6	1.9	2.0
6	1.2	2.2	2.0	1.1	1.1	2.0
7	1.1	1.9	2.0	1.3	1.7	2.0
8	1.3	1.7	2.0	1.2	1.9	2.0
9	1.5	2.1	2.0	1.4	2.1	2.0
10	1.4	1.6	2.0	1.1	1.8	2.0
MEAN±SE	1.30b±0.15[a]	1.65±0.29[b]	2.00±0.10[a]	1.33±0.16[a]	1.7b±0.27[a]	2.00±0.10[a]

Means with the same superscript letter for the same parameter for both photoautotrophic and Photomixotrophic systems and cowpea cultivars according to Duncan's Multiple Range Test are not significantly different at $P<0.05$.

DISCUSSION

Photoautotrophic micropropagation has many advantages (promotion of growth and photosynthesis, high survival percentage / smooth transition to ex-vitro environment, elimination

of morphological and physiological disorders, and little loss of plantlets due to contamination) with respect to improvement of plantlet physiology (Kozai *et al*., 1997). It has been demonstrated that plantlets can grow *in vitro* in a sugar free culture medium provided the environment is conducive to photosynthesis. Results obtained showed that the two varieties of Cowpea grew maximally under both photoautotrophic and Photomixotrophic conditions without preference. This photoautotrophic protocol (Natural Ventilation System -NVS) ensured appropriate conditions for gaseous exchange in tissue culture vessel under natural ventilation. The increased diffusion of oxygen out of the culture vessel was due to the temperature of surrounding environment. Diffusion rate of carbon dioxide into the polypropylene film at the glasshouse was higher due to influx of carbon dioxide from the environment while the relative humidity was lower in the glass house as opposed to the growth room as a result of the plastic bag coverage. These gaseous exchange conditions favoured the growth of the explants even in the absence of sucrose in the nutrient media. Probable mechanism was that the concentration gradient of the gaseous molecule, pressure and temperature gradients and velocity of air current between the inner and outer environment were maintained. Therefore, Photoautotrophic growth of *in vitro* plantlets can be significantly promoted by increasing carbon dioxide concentrations, light intensity and by decreasing the relative humidity in the culture (Chun and Kozai 2001). Forced ventilation culture systems have been developed for large-scale photoautotrophic micropropagation (Heo and Kozai, 1999), that have improved plant uniformity and have a circulating nutrients supply system (Zobayed *et al.*, 1999). Work by Jeong *et al*. (1995), Nguyen and Kozai (1998) revealed that most chlorophyllous plants *in vitro* have the ability to grow photoautotrophically but the low concentration of carbon dioxide in the air tight culture vessel during the photoperiod results in low photosynthetic rate of plants. When culture vessel with a high ventilation or a high rate of air exchange is used, the relative humidity in the vessel is reduced which in turn increases the transpiration rate (Chan and Kozai, 2001). It has also been

reported that photoautotrophic micropropagation by supplying air with carbon dioxide has been successfully used on Russet Burbank Potato and that has been recommended to seed Potato production (Pruski *et al*. 2002). Kozai et al. (1987) in their work on the epiphytic orchid, Cymbidium reported similar result. The growth rate were also increased for non-Orchid species such as *Eucalyptus camaldulensis* in photoautotrophic *in vitro* conditions and the improved plantlets quality has also increased survival rate during the acclimatization period after deflasking (Kirdmanee *et al*. 1995). Hayashi *et al* (1995) reported that the rate of air exchange and lighting cycle are important for plantlet photoautotrophic growth. Air-tight culture vessels showed lower performance than those of cotton plug capping vessels due to lack of proper ventilation. Rahman and Alsadon (2007) reported that when the culture vessels were exposed for few minutes (2/3) in front of running laminar air flow and followed by re-closing, plantlets showed better growth. This technique may allow better air flushing or ventilation occurring inside the culture vessel and improved growth of *in vitro* plantlets.

Photoautotrophic culture performed as much as photomixotrophic culture of cowpea micropropagation and as such could be utilized for large-scale production. This outcome and the development of a photoautotrophic technique, will contribute to a cost effective means of solving problems in micropropation where adequate growth environments and facilities are limited.

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