TISSUE CULTURE DERIVED PLANTLET VARIATION IN *Caladium humboldtii* Schott

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ABSTRACT

Callus cultures were initiated from corm and petiole explants of C. humboldtii on Murashige and Skoog's basal medium (MS) supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.4mg/l – 1.6mg/l) in combination with Kinetin (1mg/l) in the dark. Callus was induced on media supplemented with 0.8mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with kinetin (1mg/l) and callus induced on this media showed the best growth. Direct regeneration potential was higher in corm than in leaf explants. Regeneration was not achieved in petiole explants. De novo plant regeneration from callus cultures was not achieved and somatic embryogenesis did not yield any plantlets. Morphological differences were observed among the regenerated plantlets of C. humboldtii on Murashige and Skoog medium (MS) supplemented with 2,4-D (0.4mg/l) in combination with 1mg /l Kinetin. Polyacrylamide gel electrophoresis showed only 1 band each in the control and in the regenerants, however, the position of the bands were different. The result indicates that variation has occurred during in vitro culture. In conclusion, it has not been possible to generate plantlets from callus and it has also not been possible to advance the callus beyond the early stage of embryo development. The findings however include production of a new cultivar of C. humboldtii, initiation/growth of callus and direct regeneration of plantlets in the dark.

Keywords: *Caladium humboldtii,* variation, 2,4-D, regeneration, electrophoresis

INTRODUCTION

Caladium humboldtii Schott is an important ornamental. It is the smallest species in the genus and is known by its small size (petioles [10-24cms long], blades [5-9cm long, 2-4.5cms broad]), freely suckering habit and its lack of inflorescences (Croat and Lambert, 1986). Because of its lack of inflorescence, multiplication of this species is done vegetatively either with 'eyes' taken from its corms or by division of the plants into separate pots therefore, variation is limited or non-existent.

In the ornamental horticulture industry, however, rapid introduction of new valuable plants

to the market is very important and micropropagation is one of the methods that can be employed. In vitro cultured cells can differentiate into whole plants through organogenesis or somatic embryogenesis. Plants regenerated in this way are known to exhibit variation called 'somaclonal variation' (Larkin and Scowcroft, 1981) and the genetic stability of the explant in vitro has been said to depend both on its degree of organized structure and the influence of the aseptic environment in allowing expression of potential variability (Rice *et al.*,1992).

In *Caladium humboldtii*, natural genetic variation will not easily occur due to the fact that it is vegetatively propagated and lacks seed. Somaclonal variants will therefore be a useful source for plant improvement. Previous workers have reported micropropagation and callus formation in *Caladium* species and other plants of the Araceae family (Gliozeris *et. al.*, 2001; Tamosiunas and Gliozeris 2001; Nyman *et. al.*, 1987; Geier, 1986 and Hartman, 1974). Lecoufle (1981) also reported the discovery of a new cultivar of *Caladium humboldtii* from a population of *Caladium humboldtii* that had been tissue culture propagated but introduction of other valuable variation through somaclonal variation may be useful in programmes that are designed to improve the characteristics of the ornamental foliage plant.

In the present communication, we report the initiation of callus tissues from explants in the dark, direct regeneration of plantlets and differences of regenerated plants of *C. humboldtii* showed by electrophoresis.

MATERIALS AND METHODS

Corms of *Caladium humboldtii* Schott grown in the Screen House of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, were used as the source materials. Corm, petiole and leaf explants were washed under running tap water to remove dirt and reduce microbial population. Corms were dehusked and selected for a healthy appearance (i.e. without malformations or presence of necrotic spots). The explants were then surface sterilized with 0.7% (w/v) sodium hypochlorite solution for 10 minutes. 2 drops of Tween 20 was added to the sterilizing solution. After 10 minutes, the explants were rinsed three times in sterile distilled water. The leaves were then trimmed into pieces of about 1cm x 1cm and the corm explants into small cubes of about 1cm x1cm x 1cm, using sterilized scapel. The petioles were cut into segments of about 1cm long. The explants were cultured on full strength Murashige and Skoog's (1962) medium supplemented with three different concentrations of 2,4-D (0.4, 0.8, 1.6mg/l) each combined with 1.0mg/l kinetin and 3% (w/v) sucrose to test their effect on direct regeneration and callus initiation of corm, leaf and petiole explants. MS medium lacking growth regulators served as control. The media was solidified with 0.8% (w/v) agar (Oxoid Agar No 1, Code L11) pH adjusted to 5.7 ± 0.1 (with 0.1N HCl and/or 0.1N NaOH,) using pH meter prior to autoclaving. The medium was dispensed into 50mls Erlenmeyer flasks which were then plugged with non-absorbent cotton wool, and then further covered with aluminum foil. The medium in culture flasks were then autoclaved using Gallenkamp portable autoclave at 121°C and 15 lb/in^2 pressure for 15 minutes. Cultures were maintained at 25°C ± 2°C in the dark.

Survivors of the callus which developed were sub cultured on the same medium for about one month and were subsequently transferred to MS media supplemented with either:

1. 1mg/l, 3mg/l or 5mg/l of 6-benzyladenine (BA) in combination with 0.1mg/l 2,4, dichlorophenoxy acetic acid (2,4-D)
2. 6-furfurylamino purine (Kinetin) (1mg/l) alone, Kinetin (1mg/l) in combination with 2,4-D (0.2mg/l) or,
3. Naphthalene acetic acid (NAA) (0.1mg/l) in combination with, 1mg/l, 3mg/l or 5mg/l Kinetin.

Regenerated plants were transferred to sterilized

sawdust to acclimatize before being transferred to soil.

Electrophoresis

Proteins were extracted from fresh leaves of two control plants and three regenerated plants by homogenizing 1.6g each in a porcelain mortar in 10ml of 0.85% sodium chloride solution and were centrifuged at 10,000 revolutions per minute for fifteen minutes. The supernatant was subjected to polyacrylamide gel electrophoresis. Gels were prepared as presented in Table 1.

Four drops of each of the sample supernatants were taken in a test tube and then ten drops of glycerol, 1% 2-mecaptoethanol and sodium dodecyl sulphate (SDS) were added. These were boiled for ten minutes in a water bath to allow proper separation of chains and complete denaturation. After cooling to room temperature, a drop of 0.05% Bromophenol blue which served as a dye tracer was added to the sample, ready to be layered on the gel.

Four drops of sample were added to the top of the stacking gel layer in each of the running tubes. The tubes were placed in a column acrylamide gel apparatus with tris-glycine buffer in both upper and lower chambers, and a current of $1^1/_2$ mA was applied. The current was increased to 3 mA per gel after stacking the protein in the upper gel. When the dye front had migrated to a distance of about 1cm to the anode, the current was stopped. The gels were removed from the tubes and proteins fixed in 40% Trichloroacetic acid (TCA) for 1 hour. The gels were then stained with 0.05% Coomassie brilliant blue for 15 – 20 minutes after which destaining commenced at intervals of three hours in methanol, acetic acid and water (3:1:6) until the gels were destained. A measurement of the length of each gel and that of the band, from the top of the gel, to the middle of the band was then taken.

RESULTS

Leaf explants did not show any response to all combinations of growth regulators used. Only the combination of 0.4mg/L 2,4-D and 1mg/L kinetin elicited a response from petiole explants - creamy wet looking callus was derived (Table 2).

The results further showed that corm explants responded to all the combinations of 2,4-D (in combination with 1mg/L kinetin) employed. 33% of corms cultured on 1.6mg/l 2,4-D generated callus after 8 weeks though the callus was slow growing and did not survive. The combination of 0.8mg/L 2,4-D and 1mg/L kinetin induced a 100% callus response from the corm explants and the callus generated remained healthy for more than a year. This callus did not generate shoot primordia on different differentiating media (Table 3) even after 6wks in culture. However, for media supplemented with 1mg/L

Table 1: Upper and lower gels preparation showing the composition (volume in ml)

	Stacking Upper gel	Separating Lower gel
40% Acrylamide	1.0	10.00
Upper gel buffer 0.5M Tris-HCl pH 6.8	2.50	-
Lower gel buffer 1.5M Tris-Cl pH 8.8	-	10.00
Distilled water	6.33	19.50
10% Sodium Dodecyl sulphate (SDS)	0.10	0.40
10% Ammonium per sulphate	0.05	0.10
TEMED (N,N,N^1N^1-Tetramethylethylenediamine)	20µl	50µl

Table 2: Effect of different concentrations of 2,4-D in combination with 1mg/l Kinetin on Callus Initiation and Plant Regeneration of different explants of *C. humboldtii*

1mg/lkinetin + 2,4-D >	0.4mg/L			0.8mg/L			1.6mg/L		
Explant	Corm	Petiole	Leaf	Corm	Petiole	Leaf	Corm	Petiole	Leaf
TOR	R	C	-	C	-	-	C	-	-
%Response ± SE	33 ± 0.3	33 ± 0.3	0 ± 0	100 ± 0	0 ± 0	0 ± 0	33 ± 0.3	0 ± 0	0 ± 0
NOS ± SE	16 ± 9.3	-	-	-	-	-	-	-	-
TOI in wks	12	4	-	3	-	-	8	-	-
SOC	-	<1cm	-	>1cm	-	-	<1cm	-	-

Key: ***TOR - Type of Response*** ***NOR - No of Roots*** ***NOS - No of Shoots***

TOI *- Time of Initiation In wks* ***SE*** *– Standard Error* ***C*** *– Callus*

R *– Regeneration of plantlet* ***2,4-D*** *– 2,4,dichlorophenoxy acetic acid*

Table 3: Effect of differentiating media on induced calli

Growth Regulator	6 weeks after
$K_1N_{0.1}$	Dry callus. Cream + Green colour
$K_3N_{0.1}$	Growing callus
$K_5N_{0.1}$	3-4 Adventitious budlike growth on callus
$B_1$2,4-$D_{0.1}$	wettish callus, several adventitious budlike growth on callus
$B_3$2,4-$D_{0.1}$	Callus growing
$B_5$2,4-$D_{0.1}$	Callus growing
K_1	Proliferation of callus
K_1 2,4-$D_{0.2}$	Proliferation of callus

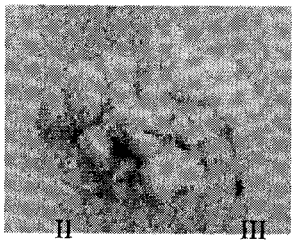
Key:
B – 6-benzyladenine
N - Naphtalene acetic acid
2,4-D - 2,4-dichlorophenoxy acetic acid
K – Kinetin
Subscript - concentration of growth regulator used in mg/l

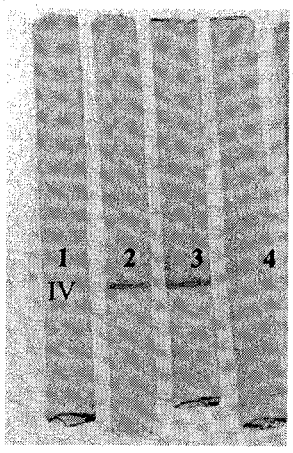
BA and 0.1mg/L 2,4-D, there was formation of adventitious shoot-like buds after 4wks in culture. Media supplemented with 5mg/L kinetin and 0.1mg/L NAA also showed signs of adventitious shoot-like buds.

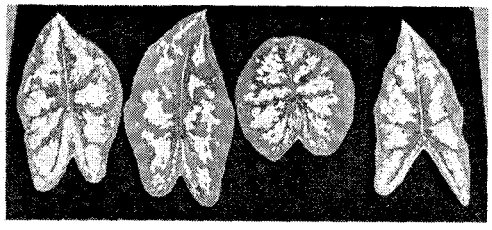
Callus from *C. humboldtii* tuber transferred to media supplemented with kinetin (1mg/l) either alone or in combination with 2,4-D (0.2mg/l) did not induce green shoot primordia rather, there was enhanced proliferation of callus instead of organogenesis. Globular stage embryo with suspensor was seen under the microscope after 3weeks of inoculating callus into embryo inducing medium. Combination of 0.4mg/L 2,4-D and 1mg/L kinetin produced 33% direct regeneration of shootlets from corms of *C. humboldtii* (Table 2). The regenerated shoots elongated further and roots developed on the same media without transfer to media devoid of phytohormones. No callus was however produced in the corm.

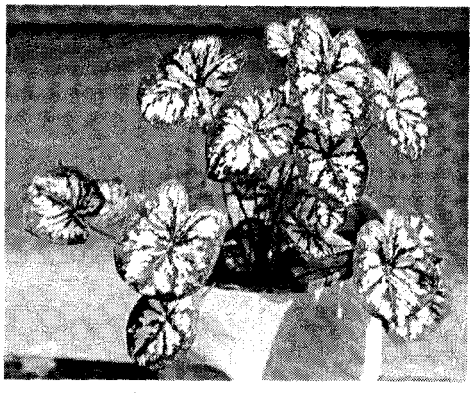
Variations

Plantlets generated from corm explant of *C. humboldtii* using 0.4mg/L 2,4-D and 1mg/L kinetin showed morphological differences in the plant's shape and colour of leaves. Polyacrylamide gel electrophoresis carried out on the leaves showed that they all had one band each









(Plate 1C). The band in *C. humboldtii* parent plant (Plate 1 C4) and the plantlet that did not differ from the parent plant were similar (Plate 1 C1). The bands in the regenerants (Plate 1 C2 & C3) looked alike and were different from both the parent and the plantlets that were similar to the parent. This was consistent with the Relative Mobility (R_M) values obtained from the measurement of the length of gels and the distance migrated by the bands (Table 4).

Table 4: Relative Mobility (R_M)

Sample	Value
A	1.6
B	1.5
C	1.5
D	1.6

Key
A – *C. humboldtii* normal plantlet
B - *C. humboldtii* plantlet with colour deviation
C - *C. humboldtii* plantlet with shape and colour deviation
D - *C. humboldtii* parent plant

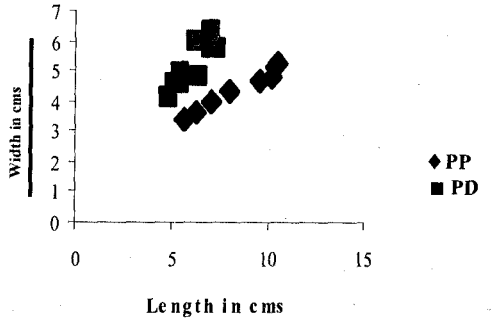


Fig. 1: Scatter diagram of leaf size of *C. humboldtii*

DISCUSSION

In the present study, 0.8mg/L 2,4-D in combination with 1mg/L kinetin was found to show 100% response for callus induction on the corm explants (Table 2), as compared to 0.4 and 1.6 mg/l 2,4-D. Since both the induction and proliferation of callus determines the effectiveness of a medium (Remotti and Loffler, 1995), 0.8mg/L 2,4-D in combination with 1mg/L kinetin is considered to be the better media supplement for callus induction from corm explants. Satish *et al.* (2003) reported similar results in *Fritillaria hupehensis* (Lilaceae), where protocormlike bodies (PLB) cultured on MS basal medium supplemented with either 0.8mg/L 2,4-D or NAA in combination with 0.5mg/L kinetin proliferated and produced both bulblets and callus. Regeneration of plantlets from callus has, however, been difficult. Formation of what looked like adventitious shoot buds was noticed on callus surface after 4 weeks of transfer to a regeneration medium containing 1mg/L BA and 0.1mg/L 2,4-D. This, however, soon disappeared as the callus changed to a watery form. Geier (1986) working on leaf segments of *Anthurium* species, reported that regeneration was highly dependent on genotype and that NH_4NO_3 level has a significant effect on callus and shoot formation.

The genetic stability of the explant *in vitro* has been said to be dependent both on its degree of organized structure and the influence of the aseptic environment in allowing expression of potential variability (Rice *et al.,* 1992) and the potential instability of adventitious regeneration has been mentioned for some monocotyledonous genera where the incidence of axillary meristems is relatively low. Polyacrylamide gel electrophoresis of the leaves from the plantlets in which morphological differences were noticed showed a difference in the location of band between the parent plant and the plantlets that indicated variation. This implies that the proteins in the mutant are different from that of the wild type. Electrophoretic separation depends on charge on the protein, the frictional coefficient of the gel material and the electric field intensity. Since the gel material and the voltage applied are the same for both the wild type and the mutant, it implies that the protein resolved in the wild type and the mutants are different. It is possible that the charge difference may be due to non- conservative amino acid replacement at the level of protein synthesis in the mutants and may in part account for the morphological differences observed in phenotypes. Since there is no seed progeny, differentiation between genetic and epigenetic variation may be difficult. The scatter diagram, however, shows a clear difference between the 2 leaf shapes (Figure 1)

Lecoufle (1981) also found a new near albino cultivar, which is completely different from the mother plant out of the several thousands of tissue

cultured *C. humboldtii* plants identical to the original. This he named *Caladium* 'Marcel'. Similar results but with *Caladium bicolor* have been reported. Ahmed *et al.* (2002), recorded variations in shape and colour pattern of regenerated plants of *C. bicolor,* Mujib *et al.* (2000) also reported chlorophyll deficiency in a sizeable number of regenerated plantlets of *C. bicolor.*

Somatic embryogenesis was not obtained in this study although globular stage embryo with attached suspensor was noticed after 3 weeks in embryo inducing medium. This suggests that the callus generated from *C. humboldtii* corm on media supplemented with 0.8mg/L 2,4-D and 1mg/L kinetin may be embryogenic.

In conclusion, this attempt to develop a protocol for the tissue culture of *C. humboldtii* and to elucidate its callus induction and plant regeneration vigour is by no means conclusive. However, it has been established that callus can be induced and maintained in the dark on MS medium supplemented with 0.8mg/L 2,4-D and 1mg/L kinetin as growth regulator. While it has not been possible to generate plantlets from callus, production of a new cultivar of *C. humbold-*

tii from direct regeneration has been achie f this variation is stable. Further studies on media manipulation in order to induce regeneration from callus and generation of embryoids through somatic embryogenesis will be in order.

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