Acclimatization and flower induction of tissue culture derived cocoyam (Xanthosoma sagittifolium Schott) plants

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Plant physiology

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Summary — Explants (shoot tips) were obtained for production of tissue culture plants, from cocoyam (*Xanthosoma sagittifolium*) plants growing in agroecological zone II (Cameroon) at the Root and Tubers Research Project (ROTREP) Biotechnology Laboratory at the Ekona Research Center. In order to utilize these tissue-culture derived plants (TCP) for field work, procedures for their acclimatization were developed. Three hundred plantlets were randomly selected from the first batch of 500 plantlets produced by ROTREP and acclimatized using 4 different growth media: i), sterilized vermiculite and sterilized top soil (SVMS, 50:50); ii), unsterilized vermiculite and unsterilized top soil (NSVMS, 50:50); iii), sterilized top soil (SS, 100%); and iv), unsterilized top soil (NSS, 100%). From the successfully acclimatized plants, 200 plants were taken for artificial flower induction studies. Gibberellic acid (GA_3) was sprayed on the tissue culture-derived cocoyam plants (TCP) at different concentrations (0, 500, 750, 1 000, 1 500 parts per million) for flower induction. There were no significant differences in growth of plantlets raised in the NSS medium and in the other growth media. Mean petiole length was in fact highest for plants acclimatized in NSS. Thus soil sterilization may not be critical for acclimatization of cocoyam plantlets. This is an important factor in the humid tropics, where resources are limited. Initial growth of plants was best for (GA_3) sprayed compared to unsprayed plants. The highest number of inflorescences as well as pollen quantity was obtained at 750 and 1 000 ppm. Growth was significantly correlated with days to bracts and spadix formation. Flowering of tissue culture derived plants occurred 20–30 days earlier than that reported for non-tissue culture derived plants.

***Xanthosoma sagittifolium* / cocoyam / malanga / inflorescence / gibberellic acid / top soil / vermiculite / acclimatization**

Résumé — Acclimatation et induction florale chez le malanga (*Xanthosoma sagittifolium*) produit à partir de culture de tissu. *Afin d'utiliser des plants obtenus par culture de tissus (TCP) pour un travail en champ, nous avons développé des procédures d'acclimatation de ces plants. Les explants de malanga* (Xanthosoma sagittifolium), *destinés à la production de plants par culture de tissus au laboratoire de biotechnologie du Centre de recherche d'Ekona (projet de recherche sur les racines et tubercules ROTREP), provenaient du Cameroun, zone agroécologique II. Trois cents plantules, parmi le premier lot de 500 produit par ROTREP, furent choisies au hasard et acclimatées en utilisant 4 différents milieux de culture:*
– vermiculite stérilisée et sol superficiel stérilisé (SVMS 50:50);
– vermiculite et sol superficiel non stériles (NSVMS 50:50);
– sol superficiel stérilisé (SS 100%);
– sol superficiel non stérile (NSS 100%).
Deux cents plantes furent prélevées parmi les plantes acclimatées pour l'étude de l'induction florale. De l'acide gibbérellique (GA_3) à différentes concentrations (0, 500, 750, 1 000, 1 500 ppm) fut pulvérisé sur

les plantules. Aucune différence de croissance significative ne fut observée entre les plantules obtenues dans les différents milieux de culture. La longueur moyenne du pétiole était cependant plus importante pour les plantules cultivées sur NSS. Nous en déduisons que la stérilisation du sol n'est pas un élément critique pour l'acclimatation de plantules de malanga. Ceci est important dans les zones tropicales humides où les ressources sont limitées. La croissance initiale des plantules fut meilleure pour les plantes traitées au GA_3 que pour les plantes non traitées. Les meilleurs résultats en nombre d'inflorescences et en quantité de pollen furent obtenus pour des doses de 750 et 1 000 ppm. La croissance fut significativement corrélée à la date d'apparition des bractées et du spadice. La floraison de plantes issues de culture de tissus eut lieu 20-30 j plus tôt que celle de plantes normales.

Xanthosoma sagittifolium = *malanga / inflorescence / acide gibbérellique / sol superficiel / vermiculite / acclimatation*

INTRODUCTION

Cocoyams (*Xanthosoma* spp and *Colocosia* spp) are an important group of tropical root crops providing energy for over 300 million people worldwide, mostly in the tropics (Lyonga and Nzietchueng, 1987). In Cameroon, cocoyams (*Xanthosoma sagittifolium*) are one of the most important root crops, only surpassed by cassava (*Manihot esculenta* Crantz) (Lyonga, 1980; Besong, 1989).

However, there has been a progressive production decline in this root crop in Cameroon and elsewhere in the tropics, attributable to a root-rot disease, the principal causal agent of which is *Pythium myriotylum* (Nzietcheung, 1985; Lyogna and Nzietchueng, 1987; Adams *et al,* 1988). Only limited success has been achieved to date in breeding for resistance against this disease due to the lack of breeding methodologies. A few clones derived from initial hybrids (Agueguia and Nzietchueng, 1984; Agueguia, 1988) have been evaluated for their resistance to the root-rot disease (Agueguia and Onokpise, 1990). Flower production still remains a severe limiting factor for cocoyam breeding and selection.

Although several researchers have reported artificial flower induction of cocoyam plants using gibberellic acid (GA_3) (McDavid and Alamu, 1976; Alamu and McDavid, 1978; Wilson, 1979; Agueguia and Nzietchueng 1984; Alamu *et al,* 1982) the varied results make it difficult to select an optimum GA_3 concentration induction.

Furthermore, virtually all the studies reported have been carried out on cocoyam clones derived from field or greenhouse-grown plants rather than tissue-culture plants. Flower induction has also been carried out during the rainy season in order to enhance flower production (Wilson, 1979; Agueguia, personal communication). This limits breeding strategy to only 1 period during the growing season. Although there have been reports on cocoyam (*Xanthosoma*) explant culture (Lalopua, 1988), little or no information exists on the acclimatization of plantlets resulting from such cultures, even though acclimatization consitutes a critical phase of tissue culture. Preliminary studies to compare the performances of plantlets placed in planticons and black plastic greenhouse pots showed better results in the latter than in the former.

The objectives of these studies were: i), to identify the most suitable media or medium for successful acclimatization of tissue culture derived cocoyam plants; ii), to induce flowering and obtain optimum gibberellic acid (GA_3) levels for flower induction of tissue culture derived cocoyam plants. To the best of our knowledge, work on artificial induction of flowers from tissue culture derived cocoyam plants has not previously been reported.

MATERIALS AND METHODS

Derivation of tissue culture plants (TCP)

Shoot tips were harvested from cocoyam plants at the 3–5 leaf stage randomly collected from various locations in the southwest province of Cameroon.

Occasionally cocoyam tubers were grown in the greenhouse for 5–6 weeks to provide young shoot tips. The average size of the shoot tips ranged from 0.5–2.5 cm. Once harvested, shoot tips were surface-sterilized by immersing them in 5% chlorox solution for 5–10 min after initial cleaning in distilled water. Following immersion in chlorox, shoot tips were rinsed in sterilized double-distilled water for 5 min.

Liquid media (Wutoh and Brooks, unpublished observations) for culturing cocoyam shoot tips was prepared by modifying the basic B5 medium of Gamborg *et al* (1968) (table I). This modified medium contained no growth regulators. For culturing, prepared liquid medium was poured into test tubes (15 × 2 cm) so that the tubes were one-third filled. The test tubes were then sterilized at 121 °C and 15 PSI for 20 min. Shoot tips (explants) were then transferred to the test tubes containing the liquid medium. Cultures were then moved into a walk-in Labconco growth chamber where they were incubated under 16 h light/8 h dark photoperiod with average day/night temperatures of 28 °C/24 °C respectively. Incubation period was 4–6 weeks, during which time the shoot tips produced cocoyam tissue culture plantlets.

Acclimatization of tissue culture-derived plants

Three hundred plantlets were randomly selected for acclimatization studies. These plantlets were then divided into 4 groups of 75 plants each and transferred directly from test tubes into perforated polythene bags (20 × 15 cm) containing the following growth substrates:

– Group I: unsterilized soil (NSS);

Table I. Composition of the cocoyam culture medium.

Compound	*Amount (g/l)*
KNO_3	2.500
$NaH_2PO_4 \cdot H_2O$	0.150
$(NH_4)2SO_4$	0.134
$MgSO_4 \cdot 7H_2O$	0.250
$CaCl_2 \cdot 2H_2O$	0.15
$MnSO_4 \cdot 2H_2O$	0.01
H_3BO_4	3×10^{-3}
$ZnSO_4 \cdot 7H_2O$	2×10^{-2}
KI	0.75
$Na_2Mo_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	2.5×10^{-5}
$CuSO_4 \cdot 7H_2O$	2.5×10^{-5}
$FeSO_4$	0.0278
Na_2EDTA	0.033
Sucrose	30

– Group II: unsterilized vermiculite + unsterilized soil (NSVMS) mixed at a ratio of 50:50;

– Group III: sterilized vermiculite + sterilized soil (SVMS) mixed at a ratio of 50:50;

– Group IV: sterilized soil (SS).

The top soil used for this study was obtained from our cocoyam germplasm nursery at Mamu, Ekona, Cameroon. Sterilization of top soil and vermiculite mixes as well as top soil alone was performed at 121 °C, 15 PSI for 15 min using prefabricated sterilized bags (70 × 40 × 60 cm).

All transferred plantlets (2-leaf-stage and 10 cm in height) in the perforated black polythene bags (20 × 10 × 15 cm) were covered with transparent plastic bags (10 × 5 × 10 cm) (to avoid wilting) and placed on a tissue culture rack where they were allowed to grow for 3 days prior to being moved outside the ROTREP biotechnology laboratory. The physical conditions on the rack were 24–28 °C under 16/8 h day/night photoperiod, while outside the laboratory the temperature was 27.5 °C with a 12/12 h photoperiod. The plants were watered every 3 days with ordinary tap water.

Flower induction of acclimatized plants

From the successfully acclimatized plants, 200 plants were taken and divided into 2 groups (A and B) of 100 plants each. Group A plants were taken to a greenhouse maintained at 29 °C under 12 h day/night conditions. The second group of plants (group B) were allowed to grow in the walk-in growth chamber at 28–30 °C under 16/8 h day/night conditions. Within each group, plants were subdivided into groups of 20 and subjected to gibberellic acid (GA_3) treatment using 5 different concentrations of 0, 500, 750, 1 000, 1 500 parts per million respectively, at the 4–5-leaf stage. This took place 7 weeks after successful acclimatization. A 1% solution of a surfactant (Tween 20) was added to GA_3 preparations as wetting agent. This concentration ensured that the GA_3 reached leaf cells without being washed off by watering or rainfall. Leaves were sprayed to the point of run-off and filling of the cup formed by the petiole bases.

All plants (groups A and B) were later transferred to 16-l pots filled with top soil and moved to an outside environment close to the ROTREP Biotechnology Laboratory. This was done 4 weeks after plants had been sprayed with GA_3. The average temperature of the outside environment was 27 °C (81 °F). Plants were fertilized at monthly intervals with available liquid fertilizer ($(NH_4)_2SO_4$, 1 000 ppm) to ensure vigorous growth.

Data collected

Survival rates, petiole length and number of leaves per plant were measured; days to bract observation, spadix production, total number of inflorescence (bracts and spadices) per plant and pollen production were recorded for data on flowering. Survival rates were determined at 2, 4, and 6 weeks after potted plants had been moved from the growth chamber to the culture rack and outside the biotechnology laboratory. Petiole length was determined biweekly by direct measurement of individual petioles from ground level to the basal lobes of the leaf. Pollen production was obtained by a score rating of 0 to 2 (0 = no pollen, 1 = profuse, 2 = very profuse). Plants were harvested for yield (cormels and corm numbers, and cormel weight) at 17 weeks after GA_3 treatment.

RESULTS AND DISCUSSION

The survival rate of acclimated plantlets was 100% in each of the 4 types of growth substrate recorded at 2, 4, and 6 weeks after transfer from test tubes. This indicates that for tissue culture cocoyam (*Xanthosoma sagittifolium*) plantlets, soil sterilization is not essential for their acclimation, especially as the unsterilized soil (NSS) provided higher mean petiole lengths (fig 1). Lalopua (1988) reported abnormalities when plants were transferred into unsterilized soil medium, and proposed that soil sterilization be carried out to eliminate soil-borne bacteria which were considered to be the principal cause of these abnormalities. However, in the present study such abnormalities were not observed.

The rate of growth was lower for plantlets raised in sterilized soil (SS) (fig 1). This may be attributed to the loss of microorganisms during heat sterilization. As a result the plant used up the existing nutrients for normal growth rate for 3 weeks, after which the nutrients became limited. The superiority of NSS can be attributed to its better water retention, sufficient nutrient availability and adequate microbial activity in spite of a slight depression regarding growth rate observed during the third week. Although a few weeds appeared on unsterilized soil mixtures (NSS and NSVMS) 2 weeks earlier than SVmSS, this did not constitute a critical problem.

The good performance of the soil/vermiculite mixture (fig 1) was probably due to the modification of the structure and texture of the soil by the addition of a soil conditioner which aids in regulation of gaseous diffusion from and into the atmosphere, retention and movement of water, and also stimulates root proliferation and development. However, due to the additional costs involved in the use of vermiculite, unsterilized soil (NSS) still remains the best alternative. This is indeed the case in regions where limited resources hamper the use of plant biotechnology procedures for crop improvement and productivity. No significant difference was observed between performance of group A and B plants.

Plants which were subjected to gibberellic acid (GA_3) treatment showed faster growth than untreated plants during the first 7–9 weeks after treatment (fig 2a, 2b). Initial increase in plant growth may have been due to a response to the growth regulator. GA_3 treatment of cocoyam has been known to result in internode elongation of cocoyam stolons

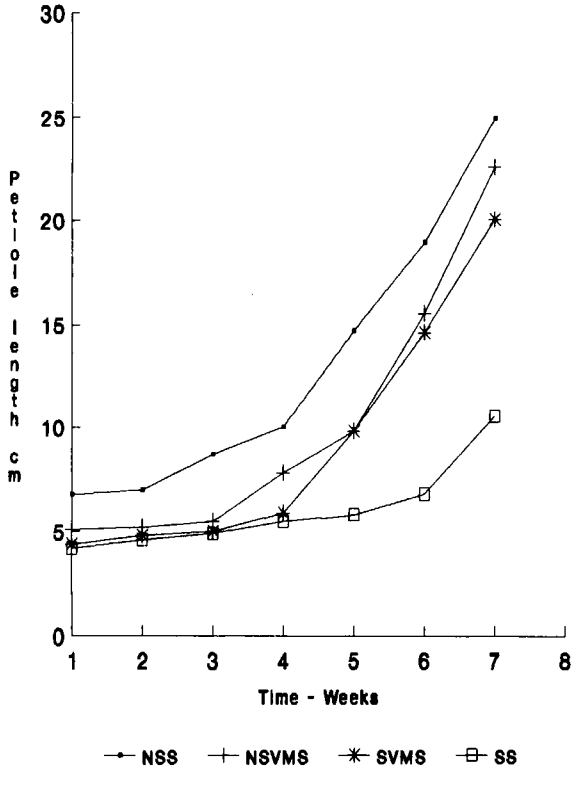

Fig 1. Effect of the different soil mixture on the growth of cocoyam plantlets. NSS = unsterilized soil; NSVMS = unsterilized vermiculite + unsterilized soil; SVMS = sterilized vermiculite + sterilized soil; SS = sterilized soil.

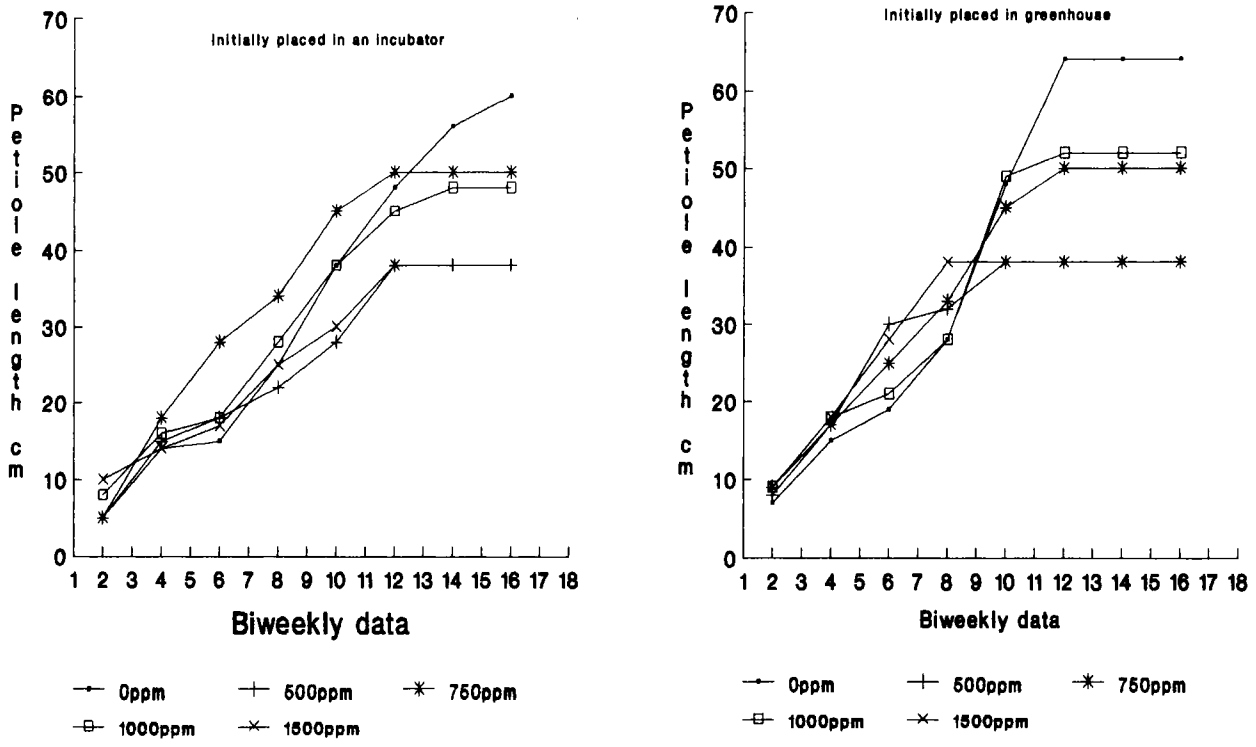

Fig 2. Effect of gibberellic acid treatment on the growth of tissue culture derived cocoyam plants. (A) Tissue culture-derived cocoyam plants initially placed in an incubator; (B) tissue culture-derived cocoyam plants initially placed in a greenhouse.

(Alamu and McDavid, 1978). Concentrations of 750 and 1 000 ppm had similar growth rates which were significantly higher than for 500 and 1 500 ppm respectively.

Days to flowering as indicated by bract and spadix formation were similar for all treatments (table II). The first indications of flowering (bracts) were observed at 50 to 82 days for all treatments and 75 to 90 days for spadix formation. Although days to bract and spadix formation were quite similar, the total number of inflorescences produced by plants treated with 750 and 1 000 ppm was significantly higher than that produced at 500 and 1 500 ppm respectively (table III). The quantity and quality of flowers produced by a plant are very important for hybridization purposes. Therefore it may be necessary to spray cocoyam plants with GA_3 at 750 and 1 000 ppm. Due to the similarity of results at these 2 concentrations, 750 ppm was chosen for economic reasons.

It should be noted that the days to the formation of reproductive structures observed in this study were 20–30 days earlier than those reported for non-tissue culture derived plants where the average number of days to flowering is 70 to 120 (Alamu and McDavid, 1978; Wilson, 1979; Alamu *et al,* 1982; Agueguia and Nzietchung, 1984). The tissue culture derived plants may have a lower endogenous level of GA_3, and spraying them with an artificial source may have provided an optimum level of gibberellic acid for vegetative and reproductive growth.

Table II. Effect of gibberellic acid (GA_3) treatment on days* to bract and spadix formation in tissue culture-derived cocoyam plants.

Gibberellic acid conc (ppm)	*TC plants outside*		*TC plants in incubators*	
	Bracts	*Spadix*	*Bracts*	*Spadix*
0	0^{a**}	0^{a}	0^{a}	0^{a}
500	50^{b}	75^{b}	60^{b}	79^{b}
750	50^{b}	75^{b}	62^{b}	75^{b}
1 000	50^{b}	75^{b}	60^{b}	75^{b}
1 500	51^{b}	90^{c}	82^{c}	90^{c}

Days on which flowering structures were first observed (mean of 20 plants). * Plants kept in old greenhouse. All plants (incubator and greenhouse) were moved to an outside environment at 27.2 °C (81 °F) at 4 weeks. ** Values within a column followed by different letters are significantly different at 5% Duncan's multiple range test.

Correlation coefficients calculated for 8 parameters (table IV) showed that the total number of cormels was significantly, but negatively correlated with the number of bracts formed and cormel weight respectively. If cormel weight is regarded as a measure of size, the fewer the cormels that are produced the bigger the cormels would be. Physiologically this is to be expected, since the accumulation of food reserves on fewer cormels increases their size (weight) compared to many centers in which these reserves might otherwise have been distributed. Positive and significant correlations were also obtained for days to spadix formation and growth. This appears to indicate that rapid vegetative growth is associated with flowering if plants have been sprayed with gibberellic acid, in particular at 750 ppm.

Table III. Total number of bracts and spadices produced 17 weeks after gibberellic acid treatment of tissue culture derived cocoyam plants.

Gibberellic acid concentration (ppm)	*TC plants* outside*		*TC plants in incubator*	
	Bracts	*Spadix*	*Bracts*	*Spadix*
0	0	0	0	0
500	9^{a**}	3^{a}	14^{a}	2^{a}
750	37^{b}	$22(2)^{b***}$	38^{b}	$20(2)^{b}$
1 000	33^{b}	$18(1)^{b}$	37^{b}	$12(1)^{c}$
1 500	18^{c}	1^{a}	14^{a}	6^{a}

* Plants kept in the greenhouse, all plants (incubator and greenhouse) were moved to an outside environment (81 °F–27.7 °C) at 4 weeks. ** Values within a column followed by different letters are significantly different at 5% Duncan's multiple range test. *** Mean pollen score in parentheses: 1 = profuse; 2 = very profuse. Scoring for pollen production was recorded for only 750 and 1 000 ppm GA-treated plants.

These studies indicate that tissue culture derived cocoyam plants provide results similar to those of non-tissue culture derived cocoyams in a similar environment. An attempt should therefore be made to utilize these new techniques so that maximum productivity is attained in these parts of the world.

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Table IV. Correlation coefficients for some vegetative, reproductive and yield characters of cocoyam treated with 750 ppm gibberellic acid.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	Db	1.00	1.00	0.89*	0.38	0.49	0.32	0.43	0.78
2	Ds		1.00	0.95*	0.39	0.37	0.35	0.41	0.43
3	Gr			1.00	0.48	0.05	0.12	0.13	0.57
4	#b				1.00	0.28	0.87*	0.71	0.27
5	#s					1.00	0.30	0.63	0.73
6	Cn						1.00	0.87*	0.50
7	Cw							1.00	0.50
8	Ps								1.00

Db = days to bract formation; Ds = days to spadix formation; Gr = growth (petiole length); #b = total number of bracts; #s = total number of spadices; Cn = cormel number; Cw = cormel weight; Ps = pollen score; * significant at $P = 0.05$.

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