EVALUATION OF GROWTH STIMULATING ACTIVITY OF ORANGE JUICE EXTRACT ON *IN VITRO* REGENERATION AND MULTIPLICATION OF COCOYAM EXPLANTS.

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Abstract
Orange juice extract was used as a growth stimulator for *in vitro* regeneration and multiplication of cocoyam plantlets. Regeneration of roots, shoots height and leaves were highest in the orange juice fortified medium compared to the control and the medium containing pure citric acid. Maximum number of leaves (30.0±3.2) and shoot height (6.2±1.4 cm) were induced in medium containing 10% concentration of orange juice while the highest numbers of roots (35.1±8.8) were induced in medium fortified with 5% orange juice concentration after 8 weeks of culture. Multiple shoot induction (8.0±1.2) was superior in the control medium compared to 7.0±0.8 and 6.5±0.6 for 10% orange juice and 2.0g/l pure citric concentrations respectively. The performances of the regenerated cocoyam plantlets in the orange juice fortified media were more robust and vigorous when compared to those induced in the control and in the medium containing pure citric acid. The significance of orange juice in *in vitro* culture is highlighted.

Key words: Orange juice, pure citric acid, cocoyam explants, regeneration, multiplication.

Introduction

Colocasia esculenta and *Xanthosoma sagittifolium* are the main edible aroids of the family *Araceae* cultivated and consumed in Nigeria. They are generally referred to as cocoyam and are about the third most cultivated staple food crop in Nigeria, covering an average land area of 661,800 ha. with a total average production figure of 3,743,200 mt. (FAO, 2005). It is estimated that roughly 400 to 500 million people in the (sub-) tropics and developing world are involved in the cultivation, consumption and trade of cocoyam (FAO, 2005). The crop (*Colocasia esculenta*) may have been domesticated in different locations from India to South China, Melanesia and Northern Australia while *Xanthosoma sagittifolium* (the new cocoyam) is hypothesized to originate in the Northern South America (Clement, 1994; Giacometti and León, 1994).

As food, *Xanthosoma* mimics yams in its various forms of usage while *Colocasia* is used mainly as a soup thickener and generally takes longer time to cook than *Xanthosoma*. Both have an acrid taste and contain raphides which can only be denatured after prolonged boiling especially *Colocasia*. *Xanthosoma* can be roasted, fried or boiled (for about 45 min) while *Colocasia* takes about 12 h or overnight to denature both its mucilage and calcium oxalate or sun dried after blanching before cooking. Cocoyam plant is also valued for its medicinal importance such as in the treatment of tuberculosis ulcers, pulmonary congestion, crippled extremities, fungal abscesses in animals, and as an anthelmintic. The stem sap is also used to treat wasp stings (Wilbert, 1986). In addition, poi, a pastry starch made from the cooked, mashed corm of the taro crop (*C. esculenta*) is credited to have the potential as a probiotic (Brown *et al*., 2004) implying that it is suitable for use in medical nutrition therapy and also shows promise in infants with allergies or failure-to-thrive.

Production challenges confronting cocoyam cultivation in the last three decades in Nigeria ranges from scarcity of quality planting materials, lack of interest by scientists and the public for cocoyam, low multiplication ratio, low genetic base and lack of improved varieties, lack of effective storage facilities and poor shelf-life of corms and cormels, declining soil fertility, as well as the incidences of pests and diseases. Traditionally, cocoyam is vegetatively propagated from tuber fragments, a practice that encourages pathogen distribution. There is considerable potential to increase cocoyam planting materials that are vigorous and disease-free through meristem tip culture. Advances in biotechnology have been of immense benefits in crop improvement programme. Through meristem tip culture technique, diseased plants can be cleaned and small pieces of explants can be used to produce thousands of plantlets in a relatively small space, time and all year round. It is possible for plant species with low levels of seed production, or seeds that do not readily germinate and plants that are difficult to propagate by cuttings to be regenerated and multiplied through this technique. Moreover, generation of useful somaclones that could be virus free, early maturing, early flowering, disease resistance, and even drought tolerance mutants are recoverable through somaclonal variation. The resulting regenerants can be multiplied and hardened for field establishment. This process is rapid, requires less time, labour, and space compared to the conventionally propagated method. Amazingly, field establishment of *in vitro* propagated plants have been observed to establish more quickly, grow more vigorously, have a shorter and more uniform production cycle and produce higher yields than the conventionally propagated propagules as they are produced under optimum environment from selected mother plants.

Sweet orange (*Citrus sinensis*) is tasty and nutritious. It is one of the major commercial fruit crops that is widely grown and consumed both as fresh fruit or juice due to its high vitamin C content and its antioxidant potential (Kiong *et al*., 2008). Erner *et al*., 1975, Goldshmidt, 1976, Einset, 1978 reported its beneficial role in promoting *in vitro* growth and differentiation of tissue culture plantlets. Nitsch, (1965), Tucker and Murashige, (1968) and Schroeder, (1972) successfully developed tissue culture plantlets from medium containing citrus juice. Murashige and Tucker, (1965) also observed that the subcultures of *C. albedo* explants from species other than *C. limon* were dependent for their growth on the supply of orange juice to the medium. A recent investigation by Erner *et al*. (1975) also revealed that most of the growth promotive activity of orange juice was dependent on citric acid which is of course, a well known component of citrus juice. Most reports on tissue culture applications of orange juice so far have been on either solanaceous or cereal crops (Azim *et al*., 2011) and on the commercial production of some economically important plants (Honda *et al*., 2001). As a matter of curiosity, we extended this orange juice approach to *in vitro* cocoyam regeneration and multiplication. Our results were exciting and the findings are as reported and discussed below.

Materials and methods

Extraction of orange juice

Riped sweet oranges (*C. sinensis*) were bought from Oye Nimo modern market, Nimo, Njikoka local government area, Anambra State, Nigeria. They were washed with clean distilled water and allowed to dry at room temperature. The oranges were later cut with sterile surgical knife and hand-squeezed. The pulps were sieved through a cheese cloth and centrifuged for 20 minutes at 2000 rpm.

Establishment of cultures

Three media were used for establishing the cultures. Medium 1 had naphthalene acetic acid (NAA) and benzylamino purine (BAP) in Murashige and Skoog (MS) complete medium substituted with 5, 10, 15 and 20% of the orange juice extract. Medium 2 had the two hormones (NAA and BAP) also substituted with 1.5, 2.0 and 2.5g/l of pure citric acid. Medium 3 is the control containing 0.1mg/l NAA and 5mg/l BAP. All the media were adjusted to pH 5.8 before adding 2 g/l of gelrite and sterilizing by autoclaving at 121^0C for 20 minutes. Four weeks old cocoyam plantlets (cultivar BL(SM/116)) were aseptically trimmed with sterile forceps and scalpel in a laminar air flow hood. The base of the explants were neatly sliced-off and inoculated singly into sterile test tubes containing 10 ml of the sterile MS media. The test tubes were sealed properly with parafilm and clearly labeled before growing the cultures at a temperature of 28 ± 2^0C for 16 h photoperiod and 8 h darkness at light intensity of 2000-3000 lux for 8 weeks.

Experimental design and data analysis

Completely randomized design (CRD) was used for the experiments. Each treatment was repeated three times and data recorded every 2 weeks after the beginning of the treatments for 2 months. Analysis of variance (ANOVA) was used to compare the number of leaves, shoots, shoot height and roots in all the media. Means were separated using DMRT test at 5% level of significance.

Results and Discussion

Different concentrations of orange juice, pure citric acid and control were investigated on *in vitro* regeneration and proliferation of cocoyam plantlets. Conventionally, MS medium containing BAP and NAA has been routinely used *in vitro* multiplication. This is probably the first time orange juice enriched medium is used as a growth stimulator in *in vitro* multiplication of cocoyam. In this study we focused our observations on four growth parameters (leaves, shoot height, shoot proliferation and root regeneration and development). We observed that there was rapid induction and development of leaves (30.0±3.2) in MS basal medium containing 10% concentration of orange juice followed closely by 15% orange juice fortified medium (28.3±8.8) after 8 weeks of the explants in culture (Table 1). A comparable response in leaf production (24.03±8.5) was recorded in the medium containing 2.0g/l of exogenous citric acid concentration, suggesting that citric acid in the orange juice may have contributed to the growth and development of the leaves. Although the orange juice fortified media were significant in leaf production at $p\leq0.05$, their degrees of contribution varies quantitatively. Thus, the highest number of leaves produced in 10% orange juice fortified medium was (30.0±3.2) compared to that of 5, 15 and 20% after 8 weeks in culture (Table 1). Comparing the triple performance of the 10% orange juice medium (30.0±3.2) on leaf production against the control (12.3±1.4), orange juice may favourable substitute BAP and NAA as a growth stimulator as it is cheaper and readily available throughout the year in the tropics. The enhanced morphogenetic effect of the orange juice on regeneration and proliferation of the explants/plantlets compared to the control and the pure citric acid enriched medium could be ascribed to the rich nutrient chemical composition of orange juice extract especially citric acid. This observation has been reported earlier by Erner *et al*., (1975) based on their extensive investigation on the partial purification of a growth factor in orange juice. Of interest is that they further observed a similar effect using exogenous citric acid on explant growth in culture. Similarly, Schroeder, (1972) obtained positive results from his work on the longevity of plant

tissues *in vitro* using orange juice. This was further confirmed by Murashige and Tucker, (1969) sequel to their discovery that the survival of subcultures of citrus albedo explants was dependent on the presence of orange juice in the medium. Similar results have also been reported by various authors on the use of fruit juices of banana, tomato, coconut, apple and pineapple in culture medium (Amo-Marco and Picazo, 1994; Siddique and Paswan 1998; Hong *et al*., 2003, Puchooa and Ramburn, 2004). As expected, the presence of additional growth factors in the juice cannot be overruled, as these may have compensated for the growth promoting differences between the performances of the explants in the optimum (10% orange juice concentration) and that of the 2.0g/l exogenous citric acid cultures (Tables 1, 2, 3 and 4). However, the possibility of these additional growth factors in orange juice has been previously reported by Murashige and Tucker, (1969), Erner, *et al*. (1975) and Einset, (1978).

Table 1: Mean number of leaves produced by the cocoyam cultivar BL(SM) 116 propagated on different growth media.

Media description	2 weeks	4 weeks	6 weeks	8 weeks
Control	3.2±0.4^{d}	8.1±0.2^{e}	10.2±0.6^{e}	12.3±1.4^{e}
MS + 5 % orange juice	2.2±0.1^{e}	5.3±0.3^{f}	7.1±0.8^{g}	10.4±0.7^{f}
MS + 10% orange juice	7.3±0.5^{a}	19.2±1.3^{a}	24.1±1.4^{a}	30.0±3.2^{a}
MS + 15 % orange juice	6.1±0.2^{b}	17.3±0.9^{b}	21.2±1.6^{b}	28.3±8.8^{b}
MS + 20 % orange juice	2.4±0.1^{e}	11.3±0.8^{d}	13.1±0.4^{d}	17.2±1.3^{d}
MS + 1.5g/l citric acid	1.2±0.1^{f}	4.0±0.2^{g}	8.6±0.4^{f}	12.01±0.3^{e}
MS +2.0g/l citric acid	4.4±0.6^{c}	12.1±1.3^{c}	17.02±6.6^{c}	24.03±8.5^{c}
MS +2. 5g/l citric acid	1.3±0.02^{f}	4.1±0.3^{g}	6.0±0.1^{h}	8.02±0.4^{g}

Key: Values represents mean ± standard errors for three replications for each treatment. Same letters are not significantly different at p≤0.05.

The patterns of growth, as well as development of the explants were observed throughout the 8 weeks period in culture. Generally, there were minimal variations in shoot heights after 8 weeks in culture with all the treatments (Table 2). However, there is a significant difference between the control and the MS medium containing 10% orange juice concentration at p≤0.05 with mean shoot heights of 5.4±0.3 and 6.7±1.4 cm respectively. Our results also provided information on shoot multiplicity of the cocoyam explants. We observed multiple inductions (Fig. 1A) of shoots in all the treatments although more of the shoots were observed in the control suggesting that this phenomenon may be genetically inherent in cocoyam (Table 3). Hence optimum shoot proliferation (8.0±1.2) was observed in the control experiment in contrast to 7.0±0.8 obtained in 10% orange juice supplemented medium (Table 3). The observed difference may be attributed to the presence of the phytohormones in the control medium especially BAP.

Table 2: Mean shoot height (cm) produced by the cocoyam cultivar BL(SM) 116 propagated on different growth media.

Media description	2 weeks	4 weeks	6 weeks	8 weeks
Control	1.8±0.4^{a}	2.7±0.3^{b}	4.9±0.4^{a}	5.4±0.3^{b}
MS + 5 % orange juice	2.0±0.1^{a}	2.6±0.2^{b}	4.7±0.3^{a}	6.1±0.4^{ab}
MS + 10% orange juice	2.0±0.3^{a}	3.6±0.4^{a}	4.3±0.1^{b}	6.7±1.4^{a}
MS + 15 % orange juice	1.4±0.1^{b}	1.8±0.1^{c}	3.9±0.2^{b}	5.2±0.4^{b}
MS + 20 % orange juice	1.1±0.1^{b}	1.8±0.2^{c}	3.9±0.3^{b}	4.1±0.3^{c}
MS + 1.5g/l citric acid	1.2±0.1^{b}	1.6±0.2^{c}	2.6±0.3^{c}	3.2±0.3^{d}
MS + 2.0g/l citric acid	1.6±0.2^{a}	2.4±0.3^{bc}	4.1±0.4^{b}	5.6±0.7^{a}
MS + 2.5g/l citric acid	0.7±0.02^{b}	1.5±0.3^{c}	3.6±0.2^{b}	4.3±0.2^{c}

Key: Values represents mean ± standard errors for three replications for each treatment. Same letters are not significantly different at p≤0.05.

Table 3: Mean number of shoots produced by the cocoyam cultivar BL(SM) 116 propagated on different growth media.

Media description	2 weeks	4 weeks	6 weeks	8 weeks
Control	2.4±0.1^{c}	5.1±0.3^{b}	7.2±0.5^{a}	8.0±1.2^{a}
MS + 5 % orange juice 'A'	3.1±0.4^{b}	5.2±0.2^{b}	5.3±0.3^{c}	6.8±0.5^{b}
MS + 10% orange juice 'B'	4.3±0.2^{a}	6.8±0.4^{a}	6.8±0.2^{a}	7.0±0.8^{b}
MS + 15 % orange juice 'C'	4.1±0.1^{a}	5.3±0.3^{b}	6.4±0.2^{b}	6.7±0.3^{b}
MS + 20 % orange juice 'D'	1.6±0.3^{c}	2.4±0.2^{c}	3.3±0.4^{e}	4.1±0.2^{c}
MS + 1.5g/l citric acid	1.2±0.3^{d}	2.3±0.1^{c}	3.8±0.3^{d}	4.1±0.2^{c}
MS + 2.0g/l citric acid	3.7±0.3^{a}	5.3±0.2^{b}	6.2±0.5^{b}	6.5±0.6^{b}
MS + 2.5g/l citric acid	1.2±0.3^{d}	2.2±0.1^{c}	2.9±0.4^{e}	3.6±0.1^{c}

Key: Values represents mean ± standard errors for three replications for each treatment. Same letters are not significantly different at p≤0.05.

In nature, roots are designed for nutrient and water uptake (Ubalua, 2010) and as a complex and dynamic organ, they control various biochemical and physiological processes that are crucial for the survival of plant (Mercier *et al*., 2001; Boru *et al*., 2003). In this study we observed that root regeneration started between 8 and 12 days of the explants in culture in all the treatments. Generally there were pronounced variations between all the treatments (Table 4). Hence at 5% orange juice concentration, root proliferation rate observed was 35.1±8.8 against 8.0±4.4 for 10% orange juice concentration and 8.2±1.2 for the control (Table 4), implying that a lesser concentration of riped sweet orange juice of 5% concentration in this study favours multiple *in vitro* proliferation of cocoyam roots (Fig. 1B). Generally, the excitement in the study was that

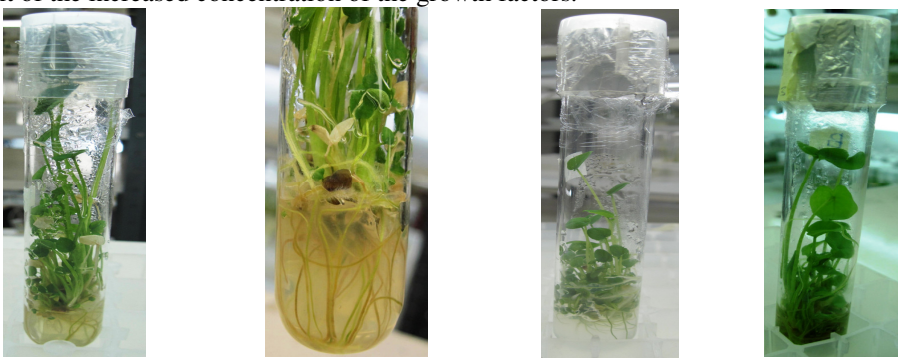
the regenerated cocoyam plantlets in the orange juice fortified media were more robust and vigorous (Fig. 1D) in contrast to those induced in the control medium (Fig. 1C).

Table 4: Mean number of roots produced by the cocoyam cultivar BL(SM) 116 propagated on different growth media.

Media description	2 weeks	4 weeks	6 weeks	8 weeks
Control	1.13±0.02^{d}	3.2±0.1^{d}	6.0±0.03^{d}	8.2±1.2^{d}
MS + 5 % orange juice 'A'	6.2±0.3^{a}	17.1±0.4^{a}	29.2±1.2^{a}	35.1±8.8^{a}
MS + 10% orange juice 'B'	1.3±0.02^{d}	2.5±0.02^{d}	5.4±0.12^{e}	8.0±4.4^{d}
MS + 15 % orange juice 'C'	0.9±0.01^{d}	2.2.±0.1^{e}	4.1±0.04^{f}	6.4±0.2^{e}
MS + 20 % orange juice 'D'	0.7±0.03^{d}	1.01±0.02^{f}	1.4±0.21^{g}	2.1±0.02^{f}
MS + 1.5g/l citric acid	5.4±0.03^{b}	8.2±2.1^{b}	12.2±1.3^{b}	15.2±4.3^{b}
MS + 2.0g/l citric acid	3.1±0.02^{c}	5.3±0.9^{c}	7.02±3.1^{c}	9.2±2.2^{c}
MS + 2.5g/l citric acid 'F'	1.2±0.03^{d}	2.6±0.4^{d}	4.1±0.2^{f}	7.6±0.3^{d}

Key: Values represents mean ± standard errors for three replications for each treatment. Same letters are not significantly different at p≤0.05.

Conclusively, lesser quantity of the orange juice yielded weaker growth response except for root induction, but beyond the optimum 10% orange juice concentration, growth and development of the plantlets were progressively inhibited probably as a result of the increased concentration of the growth factors.



A **B** **C** **D**

Fig. 1: Multiple shoot induction (A), *in vitro* root proliferation (B) Control (C); Robust and vigorous growth (D).

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