THESIS

RAPID MICROPROPAGATION OF COCOYAM (*XANTHOSOMA SAGITTIFOLIUM*) SCHOTT

Submitted By

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ANNE E. SAMA, ENTITLED "RAPID MICROPROPAGATION OF COCOYAM (*XANTHOSOMA SAGITTIFOLIUM* SCHOTT)", BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

RAPID MICROPROPAGATION OF COCOYAM

(*XANTHOSOMA SAGITTIFOLIUM* SCHOTT)

Cocoyam (*Xanthosoma sagittifolium* Schott) was rapidly micropropagated through early subcultures at four and six week intervals and division of the microshoots.

Shoot tips of approximately 3-5mm in size were used in the initiation of cultures on a modified B5 basal salt medium for six weeks. Cultures were subsequently micropropagated successfully through several procedures, which included the use of a range of growth regulator levels. Levels of 0.05μM NAA with 5, 10 and 20μM BAP as well as 1, 2 and 4μM TDZ singly or in combinations at the initiation and multiplication stages, as well as on agitated and stationary liquid media. Cultures initiation on solid media or supported on filter paper bridges was unsuccessful. However, solid media were beneficial in shoot multiplication, elongation and rooting stages. The level of 2μM TDZ alone and in combination with 20μM BAP significantly enhanced shoot proliferation, producing an average of 30 microshoots/culture, but repressed root formation. However, root initiation and development was possible in media containing only BAP at all levels tested. Shoot proliferation,

elongation and rooting continued on media devoid of plant growth regulators and was independent of the culture vessel employed.

A 100% survival of plantlets transferred into the greenhouse was achieved, irrespective of the method of acclimatization and size of the plantlet. Plantlets acclimatized in the humidity tent were less stressed, produced more and shed fewer leaves after two weeks from acclimatization. Differences in level of leaf injury were evident with control plants transferred directly to the open bench, which sustained the highest injury. The high level of survival of plantlets upon transfer to the greenhouse was attributed, in part, to the reduced numbers of stomates found on both abaxial and adaxial leaf surfaces of in vitro cultured plantlets, the high wax content and high rate of rooting.

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This thesis is dedicated to

my mother, Enanga

and

my son, Manga

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CHAPTER I

INTRODUCTION

Cocoyam, *Xanthosoma sagittifolium* (L) Schott is a monocotyledonous tuber crop species of the family Araceae. It is an important edible aroid of the tropics, and is a staple food for about 200 million people (Lyonga and Nzietchueng, 1987). Cocoyam is believed to have originated as a cultivated crop in tropical America (Onwueme, 1978) with subsequent spread to other tropical and subtropical regions. It is widely cultivated in West Africa, where it is more important than the *Colocasia* species (FAO, 1990). Although relatively new to the Pacific region, it has spread widely and rapidly, becoming an important crop in most of the islands (FAO,1990). It is equally widely cultivated in Puerto Rico, the Dominican Republic and Cuba, and has gained importance along the coastal mountains of South America, the Amazon Basin and in Central America.

The term 'cocoyam' is sometimes collectively used to refer to the two major edible ariods: *Xanthosoma sagittifolium* Schott and *Colocasia esculenta* (L) Schott. *Xanthosoma sagittifolium* is also known as tannia, new cocoyam and macabo, while *Colocasia* species are also called taro, old cocoyam, eddoe and dasheen in many parts of the world, especially in Africa (Onwueme, 1978). The similarities between these two plant species are attributed to their

environmental requirements, morphology, botany, and probably physiology in a broad sense (Onwueme, 1978). Hence, *Xanthosoma sagittifolium*, the main crop of interest in this study, will be referred to as cocoyam for convenience, as opposed to *Colocasia* species or taro.

Cocoyam, like the other tropical root and tuber crops which include taro, cassava, yam and sweet potato, constitutes a major source of energy in the diet of many people in the humid tropics, where it has served as a traditional staple food. Tropical root and tuber crops are cultivated as subsistence foods and are consumed as a basic source of low-cost calories, or as a supplement to cereals in Asia, Africa, Latin America and the Pacific. Cocoyam, like taro, is useful because it is one of the few main staples where basically all of the plant parts are consumable. Corms and cormels may be eaten boiled, mashed, pounded, or grated with soups and stews, while the youngest leaves and petioles are consumed in soups or salad.

A major importance of cocoyam is its use as an energy source during famine and disasters. FAO (1990) has shown that areas where root crops are consumed do not coincide with a high incidence of malnutrition. Although cocoyam is not among the major staple foods in the world, it, however, has considerable importance as a staple food in small and isolated communities throughout the tropics. It also contains other valuable nutrients including proteins, fats and vitamins such as β-carotene, riboflavin, niacin and ascorbic acid in small amounts, but substantial amounts of thiamine (Onwueme, 1978; Parkinson, 1984).

An additional use of cocoyam is medicinal. A decoction reduces high body temperatures in feverish persons (Ghani, 1984). Also, since the starch granules of cocoyam are very small, about one-tenth those of potato (FAO, 1990), and thus influence digestibility, cocoyam is well suited for the diets of infants and invalids. Thus, it is apparent that although cocoyam is of minor importance as compared to other major tropical root and tuber crops, it has further potential for exploitation (Okigbo, 1989). The crude protein content of some cocoyam cultivars are comparable to that of some cereals (Onokpise, personal communication).

Cocoyam is grown in a variety of production systems, mostly multiple cropping, and most importantly, it can thrive in a wide range of soils, as well as tolerate upland conditions with an annual rainfall as low as 1000 mm (Horton et al., 1984). Cocoyam is highly productive, more nutritious than cassava and quite easily reproduced (Anonymous, 1978).

In spite of cocoyam's usefulness, importance and ease of reproduction, little effort has been made to develop this crop, especially when compared to other root, tuber and cereal crops. This relative neglect by most national research institutes could be attributed to the era of colonialism, when tropical roots and tubers were considered inferior (Anonymous, 1978). This was probably associated with their poor storage ability due to high water content, which created difficulties in their marketing and processing. The breakthrough in the improvement of major grain crops which has increased world food production during the last decades has not brought similar benefits to many

developing nations, where root and tuber crops are the major staples. Most lands under production in developing nations are small isolated plots, lacking the use of inputs such as fertilizers, insecticides and machinery. Under such subsistence farming, the yields of many root and tuber crops are very low, although they have high unexploited genetic potentials for producing increased yields (Okigbo, 1989).

Of all the tropical root and tuber crops, cocoyam is the most neglected with regard to research. Emphasis has been placed on the other root and tuber crops through their recognition by international and national research institutions. Centro Internacional de Agricultura Tropical (CIAT) in Columbia emphasizes research on cassava, the Asian Vegetable and Research Development Center (AVRDC) in Taiwan focuses on sweet potato, and the International Institute for Tropical Agriculture (IITA) in Nigeria carries out research on cassava, sweet potato and yam. The International Potato Center (CIP) in Peru researches Solanum species, while the University of Hawaii focuses some efforts on taro. No comparable intensive research effort has been directed towards cocoyam, despite its importance as a staple in the diet of some regions. Currently, however, cocoyam research is gaining consideration in some agricultural development policies. For example, in 1987 USAID and the Government of the Republic of Cameroon sponsored the Tropical Roots and Tubers Research Project (ROTREP) in Cameroon to focus research efforts on cocoyam, yam and cassava. The main objectives were to produce a cocoyam cultivar that is tolerant to the root rot disease caused by

Pythium myriotylum, a major constraint in cocoyam production, and to develop a rapid propagation system for these crops.

COCOYAM PROPAGATION

The major constraints in cocoyam production include high labor and water requirements, planting materials, difficulty of breeding, diseases, pests and storage (Onwueme, 1978). Two of these constraints are of particular interest in this study: diseases and lack of a sufficient supply of quality planting stock. Most cocoyam plants are infected by Dasheen Mosaic Virus found in the leaves, corm and cormels. This virus is firmly established in Florida and other cocoyam-growing areas (Debrot and Ordosgoitti, 1974; Abo El-Nil and Zettler, 1976b). Earlier attempts to eliminate the virus involved hot water treatments of corms (Alconero, 1972), shoot tip culture (Hartman, 1974) and seed (Volin and Zettler, 1976). Success prevailed only with shoot tip culture and seed production. The development of a rapid clonal multiplication system using shoot tip culture to increase disease-free planting material would enhance increased production.

Cocoyam is conventionally propagated vegetatively by corms, cormels and suckers or daughter plants. This implies that part of the edible harvest must be reserved as planting material for the following generation. In effect, cocoyam like yam, has this disadvantage, as up to 10% of the yield is often reserved for subsequent use as planting material (Okigbo, 1989). The planting material, called 'sett', may be small corms or pieces of a larger corm; small whole cormels or sections of a large one; stem cuttings consisting of the

apical portion of the corm and lower 15-25 cm of the petiole; or daughter plants called suckers which emerge three to four months after planting. The 'sett' weighs about 125g and each corm or cormel 'sett' is such that it contains at least one bud. Of these types, stem cuttings give higher yields than corm 'setts', which in turn yield better than cormel 'setts' (Onwueme, 1978).

To obtain a 'sett', the mother plant should be mature, generally after a period of seven to twelve months depending on the cultivar. Sucker production is usually limited to two to eight suckers per mother plant per year. Suckers increase naturally by growth of axillary buds subtended within leaves or scales (Hussey, 1982). The traditional 'sett' preparation is laborious and time-consuming, regardless of the 'sett' type used. Thus, a rapid multiplication technique is needed to enhance the production process, and to help offset the problem of scarcity of quality material. Cocoyam multiplication by seed is very difficult, because flowering and consequently seed production is a rare event, except when induced with gibberellic acid (GA3) (Alamu et al., 1982; Aguegia and Nzietchueng, 1984).

Several attempts have been made using conventional procedures to more rapidly increase cocoyam planting material. One attempt to rapidly produce clonal material has been through the removal of the corm's apical bud, which exerts dominance over lateral buds. 'Setts' from the lateral buds are then produced from the corm and grown separately. In addition to this, Pardales and Dallion (1986) showed with taro that the removal of nodal scales

resulted in faster shoot development. However, this technique also increased the propagation of diseased materials.

Another attempt to rapidly increase cocoyam vegetative material while combatting disease propagation was through the usage of single bud plugs as developed by Cedeno-Maldonado and Bosques-Vega (1990). This consisted of excising small bud pieces of approximately 1.5cm in diameter, 1cm in length and weighing 4.5g, from a normal 500g corm. This piece was disinfected with 10% chlorox and treated with fungicide after drying. An advantage of this method over the traditional one is a five-fold increase in disease-free planting material. This natural vegetative multiplication provides an important means of perpetuating a unique plant with desirable characteristics.

A more efficient method to mass propagate good quality material that substantially improves production is by micropropagation. It has recently become an important and popular tool in the rapid clonal propagation of several plant species, and its application to other species is increasing. The conventional methods of speeding propagation may now be supplemented or superseded by more rapid and efficient in vitro methods.

Micropropagation is a major area of plant cell biotechnology of commercial importance (Allan, 1991). It is a means by which tissues are induced to proliferate in a manner that surpasses conventional methods. It involves the use of defined growth media supplemented with appropriate growth regulators that enable morphogenesis to occur from naturally growing

plant parts. Its significant advantage over conventional propagation methods is that a large number of plants can be produced from a single individual in a relatively short time and in little space (George and Sherrington, 1984; Debergh and Read, 1991). Micropropagation is used extensively to multiply plants with defined characteristics such as elite lines in a breeding program, and to propagate disease-free plant materials. Additional advantages include the conservation of germplasm materials that enables international transfer (George and Sherrington, 1984).

Aroid tissue culture began approximately two decades ago with the pioneering work of Mapes and Cable (1972) on taro mericloning. Hartman (1974) also used in vitro techniques to produce large numbers of pathogen-free aroids. This work was followed by that of Acheampong and Henshaw (1984) who demonstrated that cocoyam numbers can be increased tremendously in vitro, with the production of plants estimated at over one million per year from a propagule. This research indicated that cocoyam production can be enhanced through micropropagation. However, these experimental procedures are not easily duplicated, and in most cases, the rate has been as low as 1:1 or 1:2 in a hormone-free medium (Wutoh, 1989). Although it was proposed that large numbers of cocoyam could be produced in vitro, the techniques were not adequately standardized for routine micropropagation. The present study is thus undertaken with the following objectives:

- to verify and improve micropropagation of cocoyam via axillary shoot proliferation, and
- to compare the external leaf structure of tissue culture-derived plantlets and conventionally-propagated plants as regards their survival and growth in vivo.

CHAPTER II

LITERATURE REVIEW

A. Micropropagation

Micropropagation is a rapid vegetative multiplication technique using in vitro culture. It may occur via somatic embryogenesis, adventitious shoot production, or axillary shoot production (Redenbaugh, 1991). Somatic embryogenesis is the formation of embryo-like structures with a shoot and root axis; adventitious shoot formation is de novo shoot meristem formation directly from organized tissues or indirectly from callus tissue; and axillary shoot formation is the production of axillary buds and meristems from preexisting meristems. Micropropagation thus serves as an alternative means of multiplying plants, especially those that are difficult to propagate in nature.

When the optimum nutrient medium and cultural conditions are known, large numbers of shoots can be obtained from a single cultured shoot-tip within a few weeks, and the process of subculturing can produce millions of plants in a year (Debergh and Read, 1991). Micropropagation through axillary shoot formation generally results in true-to-type propagation of the parent plant. It is thus valuable for the perpetuation of plants with desired or unique characteristics, since conventional propagation may not supply sufficient propagules within a short period of time for subsequent uses.

Micropropagation also offers the possibility for easy transportation of plants in culture across borders without strict quarantine scrutiny, as well as eliminates seasonal constraints, since propagation can occur all year round (Warren, 1991).

Five different stages have been recognized in micropropagation, each of which is characterized by growth and development, and mainly controlled by the nutrients in the media as well as the surrounding conditions such as light, temperature, humidity and culture vessels (Murashige, 1974; George and Sherrington, 1984). Murashige (1974) first described three stages namely, Stages I, II and III, while Debergh and Maene (1981) emphasized the need for an additional stage designated Stage 0, for the preparation of the stock plant from which the primary explant would be derived. Stage IV was described to emphasize the importance of the transfer of plantlets ex vitro. In Stage I, the explant is established under aseptic conditions. During Stage II, multiplication of the propagules occurs. In Stage III rooting of the tissue culture-derived shoots and the beginning of the conditioning of plantlets for growth in the natural environment occurs. In Stage IV, the plantlets are established ex vitro.

1. Stage 0 - Stock Plant Selection and Preparation

The success of micropropagation has been shown to depend on the mother plant from which explants are excised. This stage was emphasized to alleviate contamination problems in vitro through the use of healthy donor plants grown to the greenhouse (Debergh and Maene, 1981). The use of

healthy plant material is equally important in the rate of survival (Debergh and Read, 1991; George and Sherrington, 1984). Debergh and Read (1991) recommended that tropical and subtropical ornamentals be maintained at a relatively high temperature such as 25°C, a relatively low humidity such as 75%, and that the watering be directed into the pot to avoid the foliage, so as to alleviate contamination problems in vitro. High quality materials are needed to multiply axenic stocks, and sometimes phytopathological monitoring is required. It is recommended that known pathogens of a crop be screened, and the cultures indexed (Cassells, 1990).

Debergh and Maene (1981) have devised a protocol for Stage 0 to reduce donor plant contamination, while Duhem et al. (1988) and Enjalric et al. (1988) proposed strategies for collecting explants from field plants. Enjalric et al. (1988) observed that contamination rates in rubber explants were higher during the rainy than the dry season. Torres (1989) indicated that levels of carbohydrates, proteins and growth substances of the mother plant would be affected by changes in the physical status, and in turn affect the response of the explant in vitro.

2. Stage I - Establishment of Aseptic Culture

The primary purpose of this stage is to establish viable axenic cultures obtained through aseptic techniques and appropriate sterilization procedures of the explant (George and Sherrington, 1984; Torres, 1989; Debergh and Read, 1991). Contamination of cultures is a serious problem in tissue culture, and it is identified to originate from microorganisms such as bacteria and

fungi, carried over on the tissue or via faulty laboratory procedures (Cassells, 1991). Campbell (1985) indicated that plant surfaces serve as habitat for microorganisms, which during plant growth and development, may enter the tissues via natural openings and wounds to colonize them. Viruses, on the other hand, are generally assisted by vectors, and become endophytic organisms. During plant tissue establishment, these surface and endophytic microorganisms have the tendency to be carried over in culture, when adequate measures are not taken to eliminate them (Tisserat, 1985; Cassells, 1991). This is largely dependent on the type of culture and explant size (Lui et al., 1988; Cassells, 1991). These researchers observed that most organisms will be generally eliminated from smaller cultures like meristematic domes, which require less sterilization time. However, larger tissues like leaf, petiole and stem explants have microorganisms that are carried over in the tissues, and necessitate adequate sterilization processes.

Some initial methods used to exclude contaminants from the explant source before disinfection include, washing with running tap water for approximately 30 minutes to two hours for explants derived from field-grown plants (Hughes, 1981); and washing with running water or soaking tissue in a mild fungicide (Torres, 1989). The different surface disinfectants used in tissue culture include ethanol, sodium hypochlorite in the form of chlorox, calcium hypochlorite, hydrogen peroxide, bromide water and mercuric chloride (Miller and Murashige, 1976; Enjalric et al., 1988; Duhem et al., 1988). These sterilants are used singly or combined, in a sequence to obtain the most

effective sterilization procedure. Sodium hypochlorite is the most commonly used sterilant. The addition of polyoxyethylene sorbitan monolaurate (Tween 20 or Tween 80) as a wetting agent, enhances the effectiveness of the sterilant by reducing surface tension between the plant tissue and water (Torres, 1989).

Most reports on cocoyam tissue culture have used apical shoots and axillary buds (Hartman, 1974; Acheampong and Henshaw, 1984; Gupta, 1985; Lui et al., 1988) of the plant just above and below ground level, with a high expectation of adhering microorganisms. Measures taken to adequately eliminate contaminants from cocoyam and taro tissues and achieve asepsis, involve the use of miniature explants (Arditti and Strauss, 1979), and sequential surface sterilization procedures (Jackson et al., 1977b; Lui et al., 1988). To obtain maximum sterilization and minimum adverse effects on cocoyam explant tissue, Lui et al. (1988) employed three changes of chlorox, separated by rinses in sterile distilled water. These researchers found the combination of chlorox and ethanol as well as the use of bactericides and fungicides inadequate and detrimental to the explants.

Cassells et al. (1988) observed that high salt and sucrose concentrations as well as the pH of the medium inhibited the growth of microorganisms, while establishing axenic cultures of *Pelargonium* x *domesticum*. They also reported that standard sterilization techniques are generally ineffective in eliminating subliminal microorganisms which pass undetected into later stages when the salt and sugar concentrations in the

medium are reduced. Such latent microorganisms are eliminated by the addition of systemic fungicides like benomyl, or antibiotics into the culture medium (Duhem et al., 1988; Enjalric et al., 1988). However, caution is needed because of their potential phytotoxicity (Debergh and Maene, 1984).

It is commonly observed that the culture medium favors the growth of both the explant and microorganisms when present, but that the latter is faster and overruns the culture medium, most often resulting in the death of the explant (Cassells, 1991; Warren, 1991). Otherwise, they render cultures unfit for subculture, or even affect the productivity of the progeny plants (Long et al., 1988; Leifert et al., 1989).

2.1. Explants

The selection of a suitable explant source is critical for the success of the technique. Differences in the ability of various cells or organs within a plant of the same genotypic constitution to regenerate in cultures has required research into the determination of the most appropriate explant (Ammirato, 1986). Debergh and Read (1991) indicated that the type of the explant required for in vitro propagation is governed to an extent by the method of shoot multiplication adopted, mostly an apical or axillary bud, although other plant parts such as leaf pieces, petioles, flower, tuber and seeds are used, and are species-dependent. Warren (1991) mentioned that an effective explant tissue is one with a high cell division potential and morphogenic plasticity, and stated that virtually all tissues in herbaceous plants could be effectively used. However, varying degrees of success is achieved depending on the culture

system used, the species in culture, and the decontamination of the culture (Torres, 1989).

Ariods have been cultured in vitro via shoot tips to produce callus and protocorm-like bodies from which plants are regenerated. Such procedures were developed for taro, cocoyam and caladium through callus cultures (Hartman, 1974). More advanced techniques for callus culture have been applied to taro (Abo El-Nil and Zettler, 1976a; Jackson et al., 1977b; Arditti, 1981; Nyman and Arditti, 1984; Yam et al., 1990a, 1990b, 1990c). Plants of *X. sagittifolium* (Acheampong and Henshaw, 1984) and *X. violaceum* (Nguyen and Nguyen, 1987) were regenerated from protocorm-like bodies initiated from shoot tips. Strauss and Arditti (1980), however, regenerated plants of *X. caracu* from callus cultures derived also from shoot tips. Procedures have been developed using other plant parts as well. Basal parts of the petioles of *X. caracu* (Staritsky, 1974), as well as taro seeds (Volin and Zettler, 1976; Jackson et al., 1977a) have likewise been employed. George and Sherrington (1984) described that, in general, the age of the donor plant, the physiological age of the explant, its developmental stage and its size, are factors of tremendous importance in determining the success of the micropropagation technique. These have not been well evaluated in cocoyam.

Shoot tip culture has proved to be an important practical method of micropropagation, which depends on stimulating axillary shoot growth by overcoming apical meristem dominance (George and Sherrington, 1984). The explants obtained from the apical portion of an intact plant consist of the

meristematic stem apex bearing several leaf initials (Bhojwani and Radzan, 1989). Large shoot tips or preformed buds are suitable to enhance axillary branching because they have a higher survival rate in culture than smaller explants, and they can resume growth faster because they retain more functionally organized tissue (Bhojwani and Razdan, 1989). Submillimeter shoot tips including the meristematic domes, are imperative when virus-free plants of cocoyam are required from one infected (Hartman, 1974; Licha et al, 1980; Lui et al, 1982, 1988; Nguyen and Nguyen, 1987).

When explants are excised from the source plant, various reactions of phenolic compounds occur and are stimulated by the mechanical injury (Debergh and Read, 1991). These involve the release of contents from broken cells, reactions in neighboring cells without any apparent symptoms of injury and/or the precocious death of some cells within the vicinity of the wound (Debergh and Read, 1991). The phenolic compounds are labile and can be easily oxidized into phytotoxic strong oxidants. This problem is alleviated by the incorporation of adsorbents such as activated charcoal or polyvinylpyrrolidone (PVP) into the culture medium, or of an antioxidant such as ascorbic acid (George and Sherrington, 1984; Tisserat,1985; Warren, 1991). Lui et al. (1988) and Abo El-Nil and Zettler (1976a) used 0.1% ascorbic acid in sterile distilled water to prevent phenolic oxidation in cocoyam.

3. Stage II - Shoot Proliferation

Shoot proliferation is a crucial stage in micropropagation, and aims at multiplying tissues that can give rise to new intact plants (George and Sherrington, 1984). The explant material in Stage I is subcultured repeatedly in Stage II to obtain plants that develop in tissue culture either through somatic embryogenesis or organogenesis (Tisserat, 1985). While embryogenesis involves plant development from embryoids, plant production through organogenesis is achieved through the production of organs, either directly from an organ explant and the subsequent enhancement of axillary shoot growth by reduction of apical dominance, or indirectly from callus or suspension cultures (Tisserat, 1985; Hussey, 1986). Under in vitro conditions, organogenesis in plants is repetitive (Ammirato, 1986) and shoot apices generate additional shoot apical meristems in leaf axils (Hussey, 1986). Ammirato (1986) and Torres (1989) indicated that excised shoot apices could be precociously induced with cytokinins to develop in vitro, by enhancing the development of quiescent or active shoot buds present. ~~large~~ This process may be repeated through several subcultures, in that as new shoots develop, they subsequently produce additional buds along their axis, which in turn are induced to develop.

Axillary shoot formation usually yields true-to-type progeny, whereas caulogenesis may yield more aberrant plants that are genetically variable (Tisserat, 1985; Hussey, 1986; Debergh and Read, 1991). However, Hussey (1986) pointed out that adventitious shoots of most plants initiated on

comparatively young tissues, seemed to be genetically homogeneous, and that totipotent tissue predominates in the young developing shoots of a number of monocotyledons. The commonly observed genetic variability from cultured tissues include variation in phenotypic expression, yield variability and loss of organogenic potential, all of which are generally due to chromosome aberrations and/or ploidy changes in chromosome number (Torres, 1989). However, of these two types of organogenesis, caulogenesis is the most desirable, because it can facilitate a rapid increase in propagules (Debergh and Read, 1991).

Hussey (1982) indicated that most monocot species with specialized organs possess a profound capacity to produce adventitious buds. This point was supported by the formation of protocorm-like bodies in cocoyam (Acheampong and Henshaw, 1984). Those bodies were regenerated into genetically stable plants. Although multiplication through axillary branching is considered the slowest type of micropropagation (Tisserat, 1985), Murashige (1974) projected a million-fold increase of *Gerbera* plantlets regenerated from a single shoot in a year. Hussey (1986) also indicated that axillary shoot cultures may continue to grow indefintely, provided they are adequately supported by the nutrients in the medium. In another study, Hussey (1977) found that cultures of *Gladiolus* cv 'Elvira' showed no apparent loss of vigor or multiplication rate, when maintained for a period as long as two years.

The means of propagation in a given system has not always been necessarily fixed. Rancillac et al. (1987) noted that the reaction of an explant in culture changes with time and/or the number of subcultures. Debergh and Maene (1981) observed that cultures yielding axillary shoots in Stage II also produced abundant adventitious shoots after a number of subcultures on the same medium.

The explant type used in Stage II is species-specific, and can be shoot clusters or the principal shoot tip (George and Sherrington, 1984). Marked differences were observed in the proliferation between apices and axillary buds in *Sitka* spruce cultures (John and Murray, 1981) as well as in *Fagus sativa, Magnolia soulangeana* and *Betula jackmontii* (Vanderschaeghe and Debergh, 1989). Hussey (1986) described two types of fragments resulting from subcultures: one type includes axillary meristem which proliferates shoots and forms clusters; the other type consists of internode tissue which gives rise to adventitious meristems.

3.1. Enhanced Axillary Branching

Axillary buds present in the leaf axils of many plant species have the potential to develop into shoots. Under natural situations, these buds remain dormant in the presence of the apical meristem for various periods. However, in order to stimulate their growth into shoots, the removal of apical dominance may be controlled by cytokinins (Bhojwani and Radzan, 1989). Hussey (1982) noted that with enhanced axillary shoot formation, success is dependent on the degree of apical dominance shown by the cultivar or

species, and that the extent to which it may be counteracted is determined by the use of adequate levels of cytokinin. Shoot multiplication will be ineffective when apical dominance is too strong with few axillary buds developing. Cytokinins stimulate axillary bud growth by releasing apical dominance. This effect has been found temporary in most instances. Lateral shoots stop growing with the diminution of the exogenous growth regulator (Debergh and Read, 1991), except for habituated tissues (Jackson and Lyndon, 1990).

Plant tissues contain suboptimal levels of growth hormones, thus in vitro growth and organogenesis largely depend on the interaction between the naturally occurring endogenous growth hormones inherent in the tissues, and their analogues incorporated into the culture medium. The rate of shoot multiplication by enhanced axillary branching in cultures can be substantially improved by growing explants in a medium containing a suitable cytokinin at an appropriate concentration with or without an auxin (Debergh and Read, 1991). These authors also noted that there is a limit to which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. However, a continuation of the shoot multiplication cycle can be achieved when the miniature axillary shoots are excised and subcultured onto a fresh medium of the same composition or hormone-free (Kerns and Meyer, 1986; Nguyen and Nguyen, 1987; Kunisaki, 1989; Yam et al., 1990a, 1990b, 1990c). This will then enable the shoot multiplication cycle to continue, and to allow a very rapid increase in plant number.

4. Stage III - Elongation and Root Induction

The primary purpose of this stage is to prepare the shoots formed in Stage II for growth in the natural environment. Shoots from Stage II are generally tiny and incapable of growth in the soil. In most circumstances, plants are elongated and rooted in a medium different from propagation medium, usually cytokinin-free (Stimart and Harbage, 1989; Yam et al., 1990c) or hormone-free (Jha and Jha, 1989; Yam et al. 1990a). This is because the cytokinin level at the multiplication stage may be carried over and can inhibit root formation. Staritsky et al. (1986) reported root formation in cocoyam, even at a relatively high BAP concentration in the multiplication medium. The reduction of macro- and micronutrient concentration during the rooting stage is frequently used. Rooting is favored in a high auxin:cytokinin ratio, and in some species, it occurs only in the complete absence of cytokinins. While light may or may not be essential, Lane (1978) and Hammerschlag (1981, 1982) showed that higher temperatures of 25-28°C generally enhanced rooting, even for plants normally grown in cool environments.

Debergh and Maene (1981) found it economical to transfer shoot clusters to a medium that ensured elongation of all shoots. However, due to the high demand of labor at this stage, it was recommended that shoot clusters be maintained until a later stage such as rooting, before separating individual plants under non-aseptic conditions (Maene and Debergh, 1985; Debergh and Read, 1991). On the other hand, Murashige et al. (1974) noted

a faster root initiation in single *Gerbera* shoots than in unseparated shoot clusters. An alternative method described by Debergh and Maene (1985) was to add liquid elongation medium to the established cultures rather than transferring them into a fresh medium. However, for many species, this alternative requires bottom cooling (Maene and Debergh, 1986; Vanderschaeghe and Debergh, 1987). McComb and Newton (1981) preferred rooting in vitro on an inert substrate, and not an agar medium. In some species like cocoyam, where roots are produced during multiple shoot formation, a separate rooting stage is unnecessary. In effect, the rooted plantlets can simply be elongated and then moved directly into the external environment for establishment.

Most commercial laboratories are replacing in vitro with ex vitro rooting. This replacement is done simultaneously with acclimatization to reduce the cost of micropropagation. Ex vitro rooting has some advantages such as the ease of transplanting non-rooted plants. Plants rooted in vitro require careful handling and are thus labor intensive. The in vitro root system is sometimes non-functioning as well (Grout and Aston, 1977a) and there is a tendency for them to be damaged during transplantation. This may result in root and stem diseases (Debergh and Read, 1991). Most of all, it is cheaper to create good rooting conditions ex vitro than in vitro. However, a method of combining in vitro and ex vitro rooting was successfully devised for apples (Welander, 1983; Zimmerman and Fordham, 1985). In effect, these researchers initiated rooting by subjecting the shoots to a sterile auxin medium with IBA or NAA

and sucrose for a period of three to seven days. These shoots were then incubated in darkness. Welander (1983) used agarized medium, while Zimmerman and Fordham (1985) used a liquid medium. In the aforementioned studies, the roots subsequently developed in a non-sterile medium during acclimatization.

5. Stage IV - Transplantation and Acclimatization

Micropropagated plantlets are initially small and heterotrophic. They must be transferred from the culture vessel to the natural environment where they must become capable of independent growth. This requires a transitional period of acclimatization. Acclimatization, or hardening is an artificial means of adaptation of plantlets from in vitro to ex vitro via the intervention of man (Brainerd and Fuchigami, 1981; Preece and Sutter, 1991). Great losses are encountered if careful stepwise procedures are not followed. Debergh and Read (1991) indicated that the procedures necessary to avoid problems at this stage are not independent, but primarily depend upon the quality and type of material produced at previous stages. Also, as Bhojwani and Radzan (1989) pointed out, the most essential requirement for successful transplantation is to maintain the plantlets under a relatively high humidity of 90-100% for the first couple of days.

The poor survival of tissue culture derived plantlets ex vitro is largely attributed to water stress resulting from excessive transpiration (Preece and Sutter, 1991) and from atypical leaf, stem and root anatomy. Debergh and Read (1991) related the susceptibility of in vitro derived plantlets to the

disorders that affect the survival of in vitro plantlets upon transfer ex vitro. These authors explained that plants transplanted from culture vessels to the greenhouse still maintain their in vitro characteristics. For example, electron microscopy has revealed atypical leaf characteristics such as reduced epicuticular wax, abnormal stomata and excessive stomata per given leaf area (Wetzstein and Sommer, 1983; Conner and Conner, 1984), abnormal leaf morphology and anatomy (Grout and Aston, 1977b; Wetzstein and Sommer, 1983), as well as improper vascular connections of the shoot and root (Grout and Aston, 1977b). Thus, these associated anomalies account for the greater water loss in tissue culture derived plants; an irregularity not encountered by conventionally propagated plants grown in lower humidities in the greenhouse.

5.1. Cuticle

The cuticle is the outermost layer on the above ground tissues of plants whose primary role is to limit water loss by transpiration. Early studies indicated marked differences between the crystalline structure of epicuticular waxes of the leaves of micropropagated plantlets and their greenhouse counterparts. These studies involved the use of naturally glaucous plants such as cauliflower (Grout, 1975), carnation (Sutter and Langhans, 1979), and cabbage (Sutter and Langhans, 1982). The in vitro derived plants lacked the characteristic crystalline wax structure of greenhouse-grown plants. However, these findings became doubtful with the appearance of some glaucous carnation plantlets in vitro (Sutter and Langhans, 1979). These glaucous plantlets with crystalline wax structure were observed on solid but

not on liquid media, and they survived acclimatization better than non-glaucous plants.

A positive correlation is reported between the amount of wax on leaves and plantlet survival out of culture. Wardle et al. (1983) and Sutter and Langhans (1982) observed increased water loss resulting from high transpiration rates in leaves of cultured plants containing reduced amounts of epicuticular wax, when compared with transpiration rates of plants grown in the greenhouse. Fuchigami et al. (1981) likewise observed different amounts of wax deposits between the abaxial and adaxial leaf surfaces of acclimatized and non-acclimatized plants.

In a subsequent study, Sutter (1985) realized that the use of scanning electron microscopy (SEM) on all plant species was inappropriate. She pointed out that except for species with a characteristic crystalline epicuticular wax, SEM was not a useful means of comparison for in vitro and greenhouse-grown glossy plants, because an amorphous waxy layer covered the epidermal surfaces. Consequently, this researcher recommended the use of gravimetric measurements as well.

In an earlier study, Grout and Aston (1977a) theorized the relationship between the environment and epicuticular wax on in vitro plants. They attributed this correlation to the high humidity within the culture vessel. Sutter and Langhans (1982) supported this theory from their observation of glaucous cabbage plantlets in vitro at a relatively low humidity of 35%. The use of dessicants to reduce the relative humidity in vitro also produced plants

with significantly increased amounts of epicuticular wax (Wardle et al., 1983). Ziv et al. (1983) found increased concentrations of agar and sucrose together with reduced relative humidity as factors that contributed to glaucous carnation shoots in vitro. Likewise, the interaction of light and temperature with relative humidity is an important factor that influences epicuticular wax deposition (Whitecross and Armstrong, 1972; Baker, 1974).

5.2. Stomata

The structure and function of stomata are other factors implicated in the rapid water imbalance exhibited by micropropagated plants upon transfer ex vitro (Wetzstein and Sommer, 1983; Conner and Conner, 1984). SEM studies indicated that the number of stomates per given surface area, as well as the stomatal structures in some cultured plant species differed significantly from those in plants grown in the greenhouse or field (Preece and Sutter, 1991). In contrast to normal elliptical and sunken guard cells, leaves of in vitro derived plants have stomata with rounded and raised guard cells in such species as sweet gum (Wetzstein and Sommer, 1983; Lee et al., 1988); red raspberry (Donnelly and Vidaver, 1984); apple (Blanke and Belcher, 1989); and rose (Capellades et al., 1990). In species such as carnation, on the other hand, stomata in plants grown both in culture and in greenhouse were similar (Sutter and Langhans, 1979). Interestingly though, the stomata of all in vitro plants observed were open (Preece and Sutter, 1991).

Considering the number of stomata per area of leaf surface of in vitro derived plantlets, greater numbers were observed in apple (Blanke and

Belcher, 1989) and rose (Capellades et al., 1990), but less on plum (Brainerd et al., 1981), as compared to their greenhouse counterparts. However, when stomatal frequency was expressed as an index per number of epidermal cells, no significant differences were seen among in vitro, acclimatized and greenhouse-grown plants (Conner and Conner, 1984).

Both relative humidity and light levels have an effect on the morphology and structure of stomata. Capellades et al. (1990) altered stomatal morphology and density in rose plants grown in vitro through an increase in light level, and a corresponding decrease in relative humidity. They obtained stomata that were depressed, more ellipsoidal and less numerous, similar to those formed under greenhouse conditions. Similar results were obtained by Lee et al. (1988) in sweet gum when only light was altered. When leaves of raspberry were removed from culture and maintained at a lower relative humidity the stomata remained raised under low light, but became sunken under high light level (Donnelly and Vidaver, 1984).

There have been several reports on the inability of stomata of cultured plants to close when first transferred from culture. An increase in abscisic acid (ABA) and carbon dioxide (CO_2) levels in plant leaves or application of mannitol characteristically results in immediate stomatal closure (Salisbury and Ross, 1985). However, when Brainerd and Fuchigami (1981) as well as Conner and Conner (1984) employed these treatments on excised micropropagated apple and *Solanum laciniatum* respectively, the expected response of stomatal closure observed with acclimatized plants was not

observed. Similar reports were made for sweet gum in the presence of ABA (Wetzstein and Sommer, 1983). Sutter and Langhans (1982) indicated that stomata closed when excised leaves of cabbage were allowed to wilt for five minutes.

Marin et al. (1988) have reported an 80% stomatal closure of excised leaves of *Prunus cerasus* when transferred from culture into a 45% relative humidity environment. Their conclusion from histochemical studies indicated the existence of a non-functional state of stomata after transfer from culture. They likewise confirmed that the new leaves formed ex vitro developed normally (Grout and Aston, 1978; Brainerd et al., 1981; Wetzstein and Sommer, 1982; Donnelly et al., 1985), and did not express the non-functional characteristic that existed in vitro. Sutter (1988) also noted the existence of substantial differences between the stomatal response of excised and intact leaves, and among various species.

Although the stomata of leaves in vitro are open, some do close with time when out of culture. Marin et al. (1988) recorded 75% stomatal closure on detached leaves of in vitro grown *P. cerasus* after 15 minutes. However, despite an hour exposure to laboratory environmental conditions, stomata of apple shoots continued to stay open when removed from culture, while 78% of those of cherry and gum plants closed (Sutter, 1988). Explaining the ineffective functioning of stomata of in vitro cultures to water loss, Sutter (1988) hypothesized that the lack of turgor necessary to maintain closure is

the result of the rapid and extreme desiccation that caused the collapse of subsidiary cells adjacent to the guard cells.

Recently, Shackel et al. (1990) showed that stomata of intact apple shoots can close in an atmosphere with a high relative humidity of 90% after a period of 12-24 hours. They, however, noted that the amount of water lost over 24 hours is equivalent to twice or thrice the initial weight of the plant. It then seems reasonable to conclude that even with closed stomata, severe water stress can occur as the result of transpirational demand.

5.3. Techniques of Acclimatization

The development of micropropagated plantlets in vitro under high humidity and low illumination results in anatomical and physiological characteristics that require their gradual acclimatization to the natural environment. Most satisfactory acclimatization techniques incur gradual changes towards lower relative humidity, higher light levels, autotrophic growth and septic environment, characteristic of the greenhouse and field (Preece and Sutter, 1991).

Although precautions are taken during acclimatization in the greenhouse or field, high rates of losses are still recorded. Plantlets can be acclimatized in vitro as well, by reducing the relative humidity (Sutter and Langhans, 1982), using a desiccant (Wardle et al., 1979; Ziv et al., 1983), increasing the agar concentration (Ziv et al., 1983), adding polyethylene glycol (Short et al., 1987; Dami, 1991), using culture vessels with semi-permeable covers (Short et al., 1987; Safadi and Hughes, 1991), uncapping the culture vessel

several days prior to transfer ex vitro (Ziv, 1986; Ripley and Preece, 1986), bottom cooling of culture vessels (Maene and Debergh, 1986; Capellades et al., 1990), or by increasing CO_2 and light in the culture vessel, coupled with no sugar in the culture medium (Kozai, 1991). Smith et al. (1990) included a triazole growth retardant, paclobutrazol, in the culture medium of chrysanthemum, to improve stomatal closure, epicuticular wax deposition on leaf and most importantly, to reduce wilting after transplantation to soil.

5.3.1. Humidity

Due to the physiological and anatomical status of in vitro derived plantlets, their maintenance at a relatively high humidity when first removed from culture is critical to ensure high survival rates. This high humidity is generally provided through the use of an automatic mist system, fogging (McCown, 1986), or a humidity chamber (Dunstan and Turner, 1984; McCown, 1986). Nevertheless, some disadvantages are associated with each of these techniques. Griffis et al. (1983) reported that with a misting system, nutrients are leached from the medium which may be too wet, thus creating an environment favorable for the growth of microorganisms. The use of fogging aids in the avoidance of most of the problems encountered with misting, except for its high cost. The relatively inexpensive humidifier may lead to heat build-up and requires monitoring (Griffis et al, 1983).

In an attempt to obtain tissue-regenerated cocoyam plants, Onokpise et al. (in press) used an inexpensive approach. They maintained a relatively high humidity via the use of transparent plastic bag covers. Staritsky et al.

(1986), however, reported that no special treatment is required to establish cocoyam plantlets in soil.

5.3.2. Film Antitranspirants

Controversial reports have been made with regard to the use of antitranspirants as a means of reducing water loss during acclimatization. Wardle et al. (1979) sprayed ABA on micropropagated cauliflower plantlets and increased stomatal resistance of the leaves. However, Sutter and Hutzell (1984) tested eight film-type antitranspirants including Folicote, on micropropagated chrysanthemum and carnation without improving plant vigor and survival. The plants were not as large and vigorous as their counterparts acclimatized in a humidity tent. Hutchinson (1984) applied Folicote to micropropagated apples which performed equally or better than those acclimatized under mist. These results indicate that the antitranspirant effect may be species-specific.

5.3.3. Light

When in vitro plantlets are exposed to low light levels, their leaves become thin, and when placed under very high light levels, they become chlorotic and scorched (Griffis et al., 1983). Hasegawa et al. (1973) recommended the gradual increase of light intensity to higher levels for asparagus plantlets. Acclimatization in the greenhouse is advantageous in that the light level is generally intermediate between those in vitro and in the field (Preece and Sutter, 1991). These authors added that the greenhouse

allows for shade, thereby resulting in reduced transpirational demand and excessive light that can be destructive to chlorophyll.

5.3.4. Temperature

Air temperature is generally controlled during acclimatization (Preece and Sutter, 1991). The heating of the greenhouse during winter months as well as cooling during summer months provide a controlled temperature suitable for growth and development. Although this control during summer months is difficult, there is an important need for a warmer medium to enhance good root activity, and to increase the humidity about the cuttings (Dunstan and Turner, 1984; McCown, 1986).

5.3.5. Soil and Containers

Some authors have emphasized the importance of the growing medium and container into which rooted plantlets are transplanted. Jones (1982) and Miller (1983) for example, argued that plantlets develop better and grow more rapidly in larger containers. It is generally agreed that the substrate should have a suitable pH, should be well buffered, reproducible and adequately porous to enable sufficient drainage and aeration (Jones, 1982; Griffis et al., 1983; McCown, 1986). It is especially important to ensure good sanitation to prevent diseases. In an attempt to identify the best suitable substrate for cocoyam transplantation, Onokpise et al. (in press) obtained 100% survival upon transferring in vitro rooted plantlets into non-sterile top soil.

6. Culture Medium

6.1. Macro- and Micro-salts

The requirement of various plant tissues and species has led to diverse major formulations such as those of Knudson (1946), Heller (1953), Murashige and Skoog (1962), Linsmaier and Skoog (1965), Nitsch (1972), Gamborg et al. (1968, 1976), Abo El-Nil and Zettler (1976a), and McCown and Lloyd (1981). Although these recipes were developed for specific tissues of a given species, many other plant species have been successfully cultured in them. For example, Murashige and Skoog (MS) macroelements have gained wide application for many plant species, although originally developed for tobacco callus. In like manner, Gamborg's B5 basal salts initially developed for soybean suspension cultures, is commonly used for culture of monocotyledons (George and Sherrington, 1984). The application of these recipes has been made possible by their integral use, and sometimes by modifications in terms of mineral ion concentration, vitamins or growth regulators.

Success in aroid tissue cultures has been made using the recipes of Murashige and Skoog, 1962 (Mapes and Cable, 1972; Hartman, 1974; Staritsky, 1974; Staritsky et al., 1986; Cedeno-Maldonado et al., 1988; Yam et al., 1990a, 1990b, 1990c), Gamborg et al., 1968 (Acheampong and Henshaw, 1984; Lui et al., 1988), Knudson C, 1946 (Mapes and Cable, 1972), Abo El-Nil and Zettler (Abo El-Nil and Zettler, 1976a), and likewise Linsmaier and Skoog, 1965 (Jackson et al., 1977b; Strauss and Arditti, 1980;

Nyman and Arditti, 1984). Although the total mineral salt composition in these mixtures is high (Pierik, 1989; Torres, 1989), the aroid tissues cultured in all cases grew and yielded adventitious tissues in the form of callus or protocorm-like bodies.

Yam et al. (1990a, 1990b, 1990c) used a basal medium containing MS microelements modified at one-tenth-strength supplemented with the auxin NAA, and taro extract to produce taro callus from lateral buds. In another study, they demonstrated that friable and active callus could only be obtained with half-strength MS basal salts supplemented with taro extract, NAA and BAP. Similarly, Staritsky et al. (1986) reported the multiplication of *Colocasia* species and *Xanthosoma* species in half-strength MS basal salts supplemented with BAP and IBA.

Several authors have demonstrated the existence of differences in the tissue culture requirements of the various aroids and even in cultivars within a species. For example, Abo El-Nil and Zettler (1976a) developed special procedures for callus induction and organ differentiation from shoot tips of taro, *Colocasia esculenta* var *antiquorum*. They developed separate media for callus initiation, maintenance, shoot differentiation, and root induction on shoots, as well as protocorm-like body formation from shoot explants using diverse levels of NAA and kinetin. In an effort to proliferate cocoyam, Lui et al. (1988) and Licha et al. (1980) compared MS, Abo El-Nil and Zettler (AZ), and Gamborg's B5 media, but only obtained a positive response with the B5 medium. In another instance, Jackson et al. (1977b) cultured shoot tips of

both taro varieties: *Colocasia esculenta* var *esculenta* and *Colocasia esculenta* var *antiquorum* on a modified Linsmaier and Skoog (LS) basal medium supplemented with IAA and kinetin. They observed that only *C. esculenta* var *antiquorum* produced callus and subsequently shoots.

6.2. Organic Substances

Vitamins constitute another important group of organic nutrients used in plant tissue culture. Since most plants are capable of synthesizing their essential vitamins in vitro (Pierik, 1989), the necessity of these substances as supplements in aroid tissue culture is equivocal. Although of varied composition in the standard media, the commonly used vitamins include thiamine (vitamin B1), pyridoxine and nicotinic acid (Acheampong and Henshaw, 1984). Myo-inositol is another essential organic component.

6.3. Complex Substances

The use of undefined complex natural products to improve growth and morphogenesis in plant tissue is a common practice. These complex products include the liquid endosperm of coconut (coconut water); fruit juices of orange, tomato, pineapple, grape; extracts of banana, taro, maize and potato; and casein hydrolysate. Of these, coconut water (CW) and taro extract (TE) are of common usage in aroid cultures. Mapes and Cable (1972) supplemented Knudson C medium with coconut water and adenine to produce protocorm-like bodies of taro, which produced plantlets when subcultured into solidified MS medium containing 10% CW. The use of TE has been indispensable for callus induction and subsequent plantlet regeneration of *C.*

esculenta var *esculenta* (Yam et al., 1990a, 1990b, 1990c). Earlier efforts to propagate this variety had not been conclusive, even on media that were successful with *C. esculenta* var *antiquorum* (Jackson et al., 1977b; Strauss and Arditti, 1980). Yam et al. (1990b) observed that TE was effective in inducing callus formation only in the presence of NAA, and that plantlet production and development were more rapid in a medium free from exogenous synthetic hormones, but containing CW and TE. However, the use of these mixtures is discouraged because of lack of knowledge of their exact composition, and especially the high variability in the composition (Murashige, 1974; Pierik, 1989). The composition of CW is known to be rich in cytokinins, and to vary with the age of the fruit, but that of TE is yet unknown.

6.4. Growth Regulators

In aroid tissue culture, the commonly used growth regulators are auxins, cytokinin and complex mixtures of different types and concentrations. Of all auxins, the most commonly employed are IAA (Hartman, 1974; Abo El-Nil and Zettler, 1976a; Jackson et al., 1977b; Nguyen and Nguyen, 1987); IBA (Staritsky, 1974); NAA (Staritsky, 1974; Abo El-Nil and Zettler, 1976a; Jackson et al., 1977b; Strauss and Arditti, 1980; Acheampong and Henshaw, 1984; Cedeno-Maldonado et al., 1988; Yam et al., 1990a; 1990b; 1990c); and 2,4-D (Abo El-Nil and Zettler, 1976a; Nyman and Arditti, 1984; Nguyen and Nguyen, 1987).) The more commonly used cytokinins are kinetin (Hartman,1974; Staritsky, 1974; Abo El-Nil and Zettler, 1976a; Jackson et

al., 1977b; Struass and Arditti, 1980; Nguyen and Nguyen, 1987; Cedeno-Maldonado et al., 1988) and BAP (Staritsky, 1974; Acheampong and Henshaw, 1984; Nguyen and Nguyen, 1987).

Other compounds with increased cytokinin activity have been discovered in the last decade. The previously used cotton defoliant, thidiazuron (TDZ), is an active cytokinin (Mok et al., 1982) and stimulates shoot proliferation in several woody species (Read et al., 1986). This finding has been implicitly supported by other researchers. Kerns and Meyer (1986) initiated shoot proliferation of *Acer* x *freemanii* with TDZ, and an interaction with BAP enhanced its effect. Gray and Benton (1991) showed that BAP and TDZ levels had similar effect on muscadine grape shoot development, except that the latter produced stunted and distorted shoots.

The specific growth regulator within any class could be important, but depends upon other medium constituents to be effective. The type of growth regulator and its concentration for micropropagation is species-dependent. Acheampong and Henshaw (1984) found BAP at 5×10^{-6} μM and NAA at 5×10^{-8} μM to be optimal for cocoyam in vitro propagation using B5 basal salts. Jackson et al. (1977b) observed in taro cultures that kinetin in LS basal medium promoted shoot development only in the presence of low levels of IAA, and that higher levels retarded root and shoot development. Research on *Xanthosoma brasiliensis* indicated that IBA and BAP stimulated the production of rooted adventitious shoots via callus formation, unlike NAA and kinetin (Staritsky, 1974).

Although Mellor and Stace-Smith (1969) did not find any appreciable effect of GA3 on potato meristem cultures, Pennazio and Redolfi (1973) as well as Novak et al. (1980) reported that 0.03mg/L GA3 in the presence of cytokinin alone or with an auxin, improved the growth of the meristem, inhibited callus proliferation, enhanced rooting and increased the amount of meristems that differentiated into shoots. However, contrary to these findings, Jarret et al., (1981) working with potato tuber discs and Murashige (1974) observed that GA3 inhibits potato shoot meristem initiation, but enhanced shoot development from the initiated meristems. GA3 plus auxin and cytokinin are sometimes added in the initiation and multiplication media of apple (Jones et al., 1977) and *Asparagus plumosus* (Fonnesbech, 1975) to enhance culture growth. Kartha et al. (1974) reported that cassava shoot initiation could be enhanced if callus derived from shoot tips was cultured on a medium containing both GA3 and BAP. The inhibitory effects of GA3 on adventitious root formation was reported by Pierik and Steegmans (1975) on stem explants of rhododendron.

6.5. Agar

The physical state of a culture medium is a critical variable, and the selection of a solid or liquid medium, shaken or stationary is not undertaken arbitrarily (Miller and Murashige, 1976). Culture media are generally in a liquid or semi-solid state, but most reports of the growth of differentiated organs in tissue culture indicate the use of solid media (Takayama and Misawa, 1981). Agar has been the common gelling agent, and the most expensive component

of solid nutrient media (Pierik, 1989). There are various types of agar such as Difco 'Bacto', 'Noble' and 'Purified' agar, Gibco Phytoagar and Flow agar exist. Debergh (1983) observed that the type of agar employed in a culture medium affects the growth and development of the tissue. Thus, he recommended that the specific type used be reported. The effects of different types of agar on excised *Picea abies* apical meristem was reported by Romberger and Tabor (1971). These researchers showed that the apical meristems grew best on Difco Purified agar than on Difco Bacto agar, and least on Difco Noble agar.

With regard to agar concentration, Murashige (1974) showed that the amount used varies with species, plant tissue, pH of the culture medium, and with the quality of the agar being employed. The quality of agar can be assessed by the amount of its organic and inorganic contaminants (Romberger and Tabor, 1971). The usual agar concentration reported is 0.6-1.0%. Stolz (1968) also observed that too high an agar concentration may be detrimental to in vitro growth, as it affects the availability of water and dissolved nutrients, particularly cytokinins (Debergh, 1983). Increased agar concentrations have, nevertheless, proved necessary in reducing vitrification (Ziv et al., 1983; von Arnold and Erikkson, 1984). Gelrite is used in some species as an agar substitute, and at concentrations approximately half of agar. However, Pasqualetto et al. (1986) noticed increased vitrification in apple with use of Gelrite.

Liquid media is widely used in callus and cell cultures, to stimulate growth and produce large cell masses. Their use in organ cultures is gaining greater importance in micropropagation due to the labor-intensive nature of solidified media (Hussey, 1986) as well as the high cost of agar. When used, liquid media are either agitated or left stationary. Agitation is facilitated by shakers to provide for good aeration, and the optimum shaking speed depends on the particular medium used, its volume and type of culture vessel (Cheng and Chua, 1982).

These researchers observed accelerated growth rates in constantly agitated or well aerated tissues. Another benefit reported for agitated media is the prevention of established polarity (Goh, 1981).

The use of liquid media generally results in faster growth (Jackson et al., 1977b; George and Sherrington, 1984). and is associated with a greater surface area exposed to the nutrients, enabling more efficient uptake. This would also disperse any toxic metabolites produced by explants, which would otherwise concentrate around it (George and Sherrington, 1984).

Miller and Murashige (1976) demonstrated the differential effects of solid and liquid media in the response of four kinds of tropical foliage plants during explant initiation and multiplication. They found that cordyline explants, for example, had equal survival on solid and liquid media, but the latter medium enhanced more shoot elongation. Low levels have proved satisfactory in vitro. For example, orchid protocorm proliferation was promoted in liquid media with a good supply of air, while shoot growth was

promoted on stationary liquid media (Cheng and Chua, 1982). Miller and Murashige (1976) obtained equally satisfactory shoot multiplication on stationary liquid medium.

The differential responses procured on solid and liquid media have been effectively combined to achieve the most efficient micropropagation systems. In some cases, solid media are used at Stage I to establish the explants, followed by transfer to a rotated or shaken liquid medium at Stage II, to promote rapid shoot growth as reported for *Lilium* species, begonias, *Saintpaulias* and gloxinias (Takayama and Misawa, 1981) and taro (Jackson et al., 1977b). The reversed procedure is true for other plant species like carnations, chrysanthemums, periwinkles (Takayama and Misawa, 1981); cocoyam (Acheampong and Henshaw, 1984); and orchids (Goh, 1981). The rapid proliferation of propagules of *Leucospermum conocarpodendron* achieved in liquid basal medium, and subsequent development into shoots when placed on filter paper bridges was described by Kunisaki (1989).

The high cost of agar and expensive shakers is being eliminated with the introduction of sterile air to increase aeration and agitation of liquid media (Goh, 1981; Cheng and Chua, 1982).

6.6. pH

The pH or hydrogen ion concentration in culture media affects growth of cultured plant tissue. It seems likely from reports on most tissues that pH within the range of 5.0-6.5 during media preparation is most suitable for

growth. Skirvin et al. (1986) pointed out that the initial pH of the culture medium is generally reduced by 0.3-0.5 units after autoclaving.

For aroid tissue culture, Arditti and Strauss (1979) recommended the pH of the culture medium to fall within the range of 5.2 and 5.8, explaining that growth might be hampered outside this range, and that agar media might not solidify at a lower pH. Butenko (1968) also mentioned additional effects of too high or too low a pH, to include the instability of IAA, GA3, Vitamin B and pantothenic acid as well as the precipitation of ammonium ions. The adverse effect of high pH on iron is averted by the common use of the chelating agent, ethylenediamine tetra-acetate (EDTA) in the culture medium (Murashige and Skoog, 1962; Street and Henshaw, 1966).

7. Environmental Factors

7.1. Temperature

Culture conditions affect growth and development, as well as determine the success or failure of the endeavor. The optimal temperature for in vitro growth and development is generally that under which the mature plants grow best ex vitro (Arditti and Strauss, 1979). In vitro temperatures are generally 3-4°C higher than under natural conditions (Pierik, 1989). Arditti and Strauss (1979) indicated that temperatures between 20-27°C are optimal for aroid tissue culture. Lane (1978) and Hammerschlag (1982) noticed that higher temperatures of 25-28°C enhanced root formation of apple and plum, respectively, although these plants normally grow under cool environments.

7.2. Light

Plants depend on light for photosynthesis, but the photosynthetic rate of plant tissues in vitro is relatively low. Light duration, quality, and intensity affect the photosynthesis necessary for growth and development (Arditti and Strauss, 1979; George and Sherrington, 1984). For aroid shoot tip culture, light duration used has varied from 12-24 hours (Mapes and Cable, 1972; Hartman, 1974; Acheampong and Henshaw, 1984). Yam et al. (1990a, 1990b, 1990c) initiated callus cultures of taro, *C. esculenta* var *esculenta* with a 16 hour photoperiod provided by a mixture of Gro-lux and incandescent bulbs, but darkness was also used to initiate and grow callus cultures of *C. esculenta* var *antiquorum* (Strauss and Arditti, 1980). The quality of light is largely provided by cool white fluorescent and incandescent bulbs, but the former is preferred because it produces blue and red light wavelengths necessary for plant growth and morphogenesis, as well as generates less heat (Arditti and Strauss, 1979; George and Sherrington, 1984). Although the light intensity for micropropagated cultures is recommended to increase from one stage to another, reports on cocoyam indicated that less than 10,000 lux (Hartman, 1974) are used. Foliage plants of some tropical species multiplied most rapidly in 3,000 to 10,000 lux (Miller and Murashige, 1976).

CHAPTER III

MATERIALS AND METHODS

1. Source and Excision of Explants

Xanthosoma sagittifolium cv South Dade White plants were obtained from the Tropical Fruit Co. in Homestead, Florida, as sprouted corm sections. Each corm section was potted in 10cm polyethylene pots in a soilless mix of peat, perlite and vermiculite (1:1:0.5) (v:v:v). These plants were planted in September 1991 and maintained in a greenhouse under natural photoperiods. Heating was provided during the winter months, and cooling during summer months, to maintain 23 $\pm$ 2°C. Plants were watered as needed with tap water and twice weekly with liquid fertilizer containing N:P:K at 20:10:20 (v:v:v).

At approximately eight weeks after planting, sprouts of about 10cm high were collected, trimmed with a scapel to approximately five centimeters and washed under running tap water for 30-60 minutes. These were further excised to finally obtain shoot-tips of 3-5 mm by removal of the outermost leaves. The shoot tip comprised the apical meristem with 4-6 leaf primordia, and approximately 0.5mm of corm tissue at the base. Each sprout provided only one shoot tip from the growing point. Shoot tips could not be trimmed

to provide uniform sizes, as the sizes reduced unequally with the removal of the outermost leaves.

In a laminar flow hood, the excised explants were then subjected to disinfestation according to the sequential surface sterilization procedure described in Lui et al. (1988). These explants were first immersed in 0.53% sodium hypochlorite containing 0.01% Tween 20, then into 0.25% and 0.05% sodium hypochlorite for ten, five and one to two minutes, respectively. Each sterilization procedure was followed by three rinses in sterile distilled water containing filter-sterilized 0.1% ascorbic acid. Explants were then blotted onto sterile filter paper before transferring into the culture medium.

2. Basal Medium

Throughout this study, the basal medium (BM) employed was a modified Gamborg's B5 (Gamborg et al., 1968) mineral salts and vitamins supplemented with 0.05μM NAA, unless specified otherwise. The modified component of B5 micro-salts was $MnSO_4.4H_2O$ at 10mg/L. Organics consisted of myo-inositol (100 mg/L), thiamine HCl (10 mg/L), nicotinic acid (1 mg/L) and pyridoxine HCl (10 mg/L). Sucrose at a concentration of 30g/L was provided in all cases as the source of carbon or energy. Whenever a semi-solid medium was desirable, agar (Sigma Agar, Type A) was added at a concentration of 0.4%, after pH adjustments to 5.7 $\pm$ 0.02. Agar was dissolved by bringing the medium to approximate boiling point. Aliquots of media were then dispensed into culture vessels.

Inorganic and organic substances were prepared as stock solutions of 10 or 100 times the required concentration and were maintained under refrigeration. Aliquots of 100ml and 10ml, respectively, were taken from each stock solution per liter of medium to provide the required concentration. The relative insolubility of thidiazuron (TDZ) was resolved by its dissolution in equal quantities of water and dimethyl sulfoxide (DMSO). Hence, the final medium containing TDZ also contained approximately 0.01% DMSO.

Erlenmeyer flasks (125ml) and test tubes (25 x 150mm) were employed for growing cultures. Aliquots of 25ml and 15ml were dispensed into the flasks and test tubes, respectively. In order to hold moisture, reduce infection and allow for gaseous exchange, flasks containing culture media were first stoppered with non-absorbent cotton plugs, and then covered with aluminium foil. Test tubes were covered with polypropylene closures, Kaput caps (Bellco Glass, Inc., N.J.). The media-containing vessels were then autoclaved for 18 minutes at 121°C.

3. Explant Establishment and Multiplication

In an attempt to obtain the most efficient micropropagation procedure, various experiments were carried out concurrently. In vitro induction of morphogenesis was accomplished by manipulating growth regulators, singly or in combinations. The auxin, naphthaleneacetic acid (NAA) plus two cytokinins, N^6-benzylaminopurine (BAP) and thidiazuron (TDZ) were the most commonly employed growth regulators.

For the different experiments, various treatments were employed at different concentrations of the two cytokinins. In some experiments, explant initiation medium was essentially the same as that used for shoot multiplication, and in other cases, the cytokinin level was increased in the latter stage. At each multiplication phase shoot clusters were subcultured at six week intervals for proliferation of shoots. Shoot elongation and rooting was carried out simultaneously either in the BM and/or a hormone-free medium. Some shoots were rooted in BM supplemented with 5μM BAP alone, and in addition to 0.05μM GA3.

During enhanced shoot multiplication, tiny shoots (<2mm) which formed could not be separated into single shoots. A minimum mass of tissue bearing 3-6 new axillary microshoots, cut vertically with some part of the basal tissue attached to the piece, constituted the explant used to enhance propagation. In preparing propagules for elongation, rooting and subsequent transfer to soil, shoot clusters were divided into single shoots and recultured. All pretransplant culture media were solidified with agar.

In each of the experiments percentage of contamination was scored by counting the number of cultures with bacteria or fungi growing in them after four to six weeks of culture. Survival was assessed as the percentage of cultures that were contaminant-free and increased in size for subsequent transfer into the next phase after six weeks.

A randomize complete block design was used in each experiment. All data were subjected to an analysis of variance using unequal replications

where contamination was observed. Treatment means were separated by Tukey's Multiple Range Test at a 5% level of significance. Statistical analysis were done with the STATGRAPHICS computer package.

4. Culture Conditions

All cultures were incubated in growth rooms maintained at 25 $\pm$ 3°C, under continuous illumination. These culture conditions were the same for each of the different stages, with slight differences associated with location within the growth room. Lighting was supplied by cool white fluorescent tubes at an intensity of 60-100 μmol/m^2/s. Light intensities were measured with an LI-185 Quantum/Radiometer/Photometer (Lambda Instruments Corp., Lincoln, Nebraska).

A. MEDIA SUBSTRATE INFLUENCE ON COCOYAM CULTURE

In an attempt to study the effect of different media substrates, solid or liquid, explants were initiated on three media supplemented with either 5μM BAP, 20μM BAP or 2μM TDZ. Each medium was either solidified with 0.4% agar or maintained in the liquid state. Liquid media were either continuously shaken on a rotary shaker (Model New Brunswick Scientific, Edison, N.J.) at 80 rpm; held stationary but with the suspension of the explant in the medium; or held stationary with the tissue supported on a filter paper (Whatman #1) bridge. Cultures were maintained in flasks, each of which served as a replicate. The 12 different treatments were replicated 10 times, and the experiment was repeated once. Cultures were observed at two-week intervals and scored from 1 to 4 for survival frequency and shoot elongation. The

rating scale was as follows: 1 - creamy or dead cultures showing no apparent growth; 2 - growth initiation and appearance of green coloration; 3 - increase in growth, green coloration and leaf differentiation; 4 - development of healthy green leaves.

Six week old cultures were subcultured into various multiplication media. All live cultures were transferred to liquid basal media with various hormones. Cultures initially on the shaker, as well as 5μM and 20μM BAP from stationary liquid were maintained in their respective treatments, while 2μM TDZ from stationary liquid was reduced to 1μM TDZ. Observations were made twice after two-week intervals, with respect to the number of axillary shoots formed.

B. THIDIAZURON INFLUENCE ON MULTIPLICATION

In an initiation experiment, the BM was supplemented with TDZ at levels of 1μM, 2μM, 4μM and 8μM, as well as with 5μM BAP which served as the control. Test tubes of stationary liquid media, without any form of support, was used in all cases. The treatments were replicated 10 times. This particular experiment employed two sizes of explants: 3-5mm or 6-10mm. Data was collected every two weeks for six weeks, and included shoot length, base diameter and number of axillary shoots as well as roots formed per culture.

C. INTERACTION OF TDZ AND BAP ON COCOYAM CULTURE

In a continued effort to investigate the most efficient micropropagation procedure, the explants were cultured by the following methods.

1. Initiation of Cultures on Agitated Liquid Media

Cultures were initiated in six treatments of BAP at levels of 0μM, 10μM and 20μM, factorially combined with TDZ levels of 0μM and 2μM and supplemented with 0.05μM NAA. These liquid cultures were constantly rotated on a rotary shaker (Lab-Line Orbit Shaker No. 3590, Lab-Line Instruments, Inc., Melrose Park, Illinois) at 80 rpm. The cultures were replicated 21 times and were maintained in their various initiation media for six weeks. They were observed at two-week intervals for shoot length, base diameter, axillary shoots and adventitious root formation.

The shoots obtained at the end of the initiation phase were then trimmed of any axillary shoots to ensure uniformity, and transferred into two media for proliferation. These multiplication media consisted of BM supplemented with either 20μM BAP or 2μM TDZ. Shoots from each of the six initial treatments were subsequently cultured factorially into these multiplication media, thus constituting 12 treatments at this stage. Cultures were observed for eight weeks, principally for the formation of axillary shoots and adventitious roots, but data were also collected for shoot length and base diameter.

A second subculture was made into fresh media at the end of this period with microshoots serving as explants. Cultures were either maintained in their respective treatments on shakers, or subcultured into semi-solid media in test tubes. The latter cultures were derived from the 2μM TDZ treatment only. Cultures in the semi-solid media were treated in two different ways.

They were either maintained in the same media, or were subcultured into one that was hormone-free. At the end of an incubation period of six weeks, microshoots from the semi-solid media were subsequently subcultured into BM and hormone-free media contained in flasks and test tubes. The intent of this was to evaluate the effect of container size and absence of growth regulators on proliferation. Cultures were incubated for four weeks, and data were collected on shoot and root formation at two-week intervals.

2. Initiation of Cultures on Stationary Liquid Media in Test Tubes

In this experiment, the source material was six-week-old tissue culture-regenerated plants grown in the greenhouse. Shoot-tip explants were directly initiated in various liquid media that served for multiplication as well. The test media consisted of BM supplemented with BAP at 5μM and 10μM, in factorial combinations with 0μM, 1μM, 2μM and 4μM TDZ. A no-cytokinin control treatment was also included. The cultures were in test tubes and observed at two-week intervals. Data were collected on the number of shoots and roots per culture, main shoot length as well as the percentage of cultures that rooted. One subculture was made into the same respective media and grown for six weeks. At the end of this period, a second subculture for four weeks was made onto BM and hormone-free semi-solid media. Data were collected as before.

3. Initiation of Cultures on Stationary Liquid Media in Erlenmeyer Flasks

A final initiation procedure was tested and consisted of establishing shoot-tip explants in a uniform medium for a month. This medium consisted

of BM supplemented with 5μM BAP (Acheampong and Henshaw, 1984). The shoots were then transferred into various treatments of BAP and TDZ levels, singly or in combinations for multiplication. In this case, BAP levels of 0μM, 5μM, 10μM and 20μM were factorially combined with TDZ at 0μM and 2μM. The media were all stationary liquid contained in Erlenmeyer flasks. Cultures were maintained in their respective media for a period of six weeks, before reculturing into fresh media for another six-week period. A subsequent subculture was carried out into the same multiplication media, as well as into one without hormones in test tubes. Cultures were observed for axillary shoot proliferation and adventitious root formation.

D. BEHAVIOUR OF COCOYAM PLANTLETS IN THE GREENHOUSE FOLLOWING VARIOUS ACCLIMATIZATION TECHNIQUES

Acclimatization studies were carried out during both winter and summer. In both seasons, virtually the same procedure was undertaken with slight modifications as noted.

Plants used for acclimatization were previously proliferated in vitro in 2μM TDZ multiplication medium. For summer studies individual shoots were then elongated and rooted in BM, whereas media supplemented with 5μM BAP alone or 5μM BAP plus 0.05μM GA3 were used with shoot clusters for the winter studies. After a culture period of four weeks (summer) and six weeks (winter), vigorous plants of approximately 7cm (summer) and 3cm (winter) tall, with about four leaves and an average of seven roots per plant, were taken out for transplantation. Agar was gently washed off the roots

with tepid tap water. Plants were then transplanted into 10cm plastic pots containing premoistened non-sterile soilless substrate composed of peat, perlite and vermiculite at a ratio of 1:1:0.5 (v:v:v). During transplantation the number of leaves and roots and plant height were recorded for each plant. Plant height was measured from the basal plate to the lamina tip of the youngest fully expanded leaf. The transplants were then subjected to four different acclimatization treatments for five days as follows: (a) no acclimatization (control); (b) mist; (c) humidity tent; and (d) test tube acclimatization by uncapping. Each treatment had at least 42 plants.

Plants for the control treatment were transferred directly to an open bench in a shaded greenhouse. This was done in the evening, on a day that was cloudy and cool, even in summer. The initial temperature and relative humidity were 25°C and 63%, respectively. A record of greenhouse conditions after transplantation, using a hygro-thermogragh (Model Belfort Instrument Company), indicated high and low temperatures averaging 26°C and 16°C, respectively, with corresponding relative humidities of 54% and 94% over the period of observation. The average greenhouse conditions during winter were a relative humidity of 40 $\pm$ 5%, 22 $\pm$ 3°C, and light intensities of approximately 400 μmol/m^2/s.

The second treatment of plants was placed under an automatic misting system set for six seconds at intervals of eight minutes. In order to gradually reduce the humidity, the misting interval was increased to 16 minutes after the first two days for the remaining three days of acclimatization.

A locally constructed plastic covered humidity chamber with the dimension of 127 x 92 x 62cm (Figure 1) was used to acclimatize the third set of plants. The humidity was provided with the aid of a humidifier. Initial relative humidity was 98% with a temperature of 25°C. The relative humidity was gradually reduced after the second day of acclimatization by partially and fully opening the flaps of the chamber. On days three and four, the outermost flap was opened half-way and fully, respectively, thus lowering the relative humidity to 96% and 94%. On the fifth day, the innermost flap was fully opened, and the relative humidity was maintained at 92%.

The fourth acclimatization treatment was not included in the winter acclimatization. This treatment was conducted in culture vessels by partial uncapping. The cultures were placed on a bench in the same environment as the control plants. Caps were loosely opened for the first two days, and then totally removed for the remaining three days of acclimatization to expose the plants to the natural environment while in the test tubes. After five days of acclimatization in the test tubes, the plants were taken out, agar gently washed off the roots, and then transplanted into the soilless mix as previously described.

At the end of acclimatization, plants were transferred to an open bench and grown under standard greenhouse conditions. Plant survival was assessed by the percentage of damaged leaves (number of damaged leaves x 100 / total number of leaves). Wilting assessment was determined according to the condition of the leaf on a scale of 0 - 4 as follows: 0 -

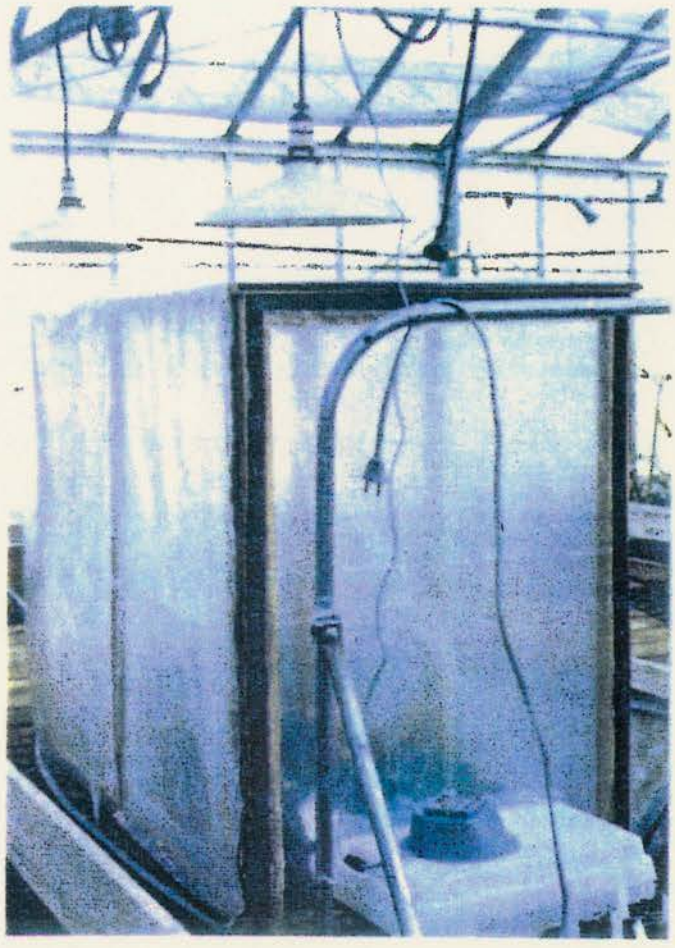


Figure 1. Humidity tent used to acclimatize cocoyam plantlets upon transfer ex vitro.

complete wilting and shrivelling of all leaves (dead); 1 - partial wilting and shrivelling of all leaf laminae; 2 - partial wilting and shrivelling of 2 - 3 leaf laminae; 3 - partial wilting and shrivelling of only 1 leaf lamina; and 4 - fully turgid intact leaves showing no wilting. These observations were made at one hour, one day and then weekly for six weeks after acclimatization. Further measurements of plant height and number of leaves formed and shed were made just after acclimatization at weekly intervals.

The experiment was completely randomized. Data were subjected to an analysis of variance, and means were separated with Tukey's Multiple Range Test at the 5% level of significance.

E. COMPARATIVE STUDY OF NUMBER OF STOMATES IN TISSUE CULTURE-DERIVED AND CONVENTIONALLY-PROPAGATED PLANTS

Stomates were examined to ascertain their function and influence relative to plant survival upon transfer from in vitro to natural conditions.

Leaf samples were obtained from vigorous plants of three sources as follows: (a) five-week old in vitro plants ready for transplantation from culture; (b) six-week old acclimatized plants growing under greenhouse conditions; and (c) conventionally propagated plants grown and maintained under greenhouse conditions. The youngest fully-expanded leaves were collected from 10 different plants per source.

Leaf impressions were made using a thin film of transparent fingernail polish (Capellades et al., 1990; Dami, 1991). This was applied to peripheral sections on either side of the midrib and on both abaxial and adaxial surfaces

of the lamina. After the fingernail polish was allowed to dry for approximately five minutes, the epidermal cell layer was peeled with a transparent adhesive tape. The imprints were then placed on microscope slides for observations.

Stomata and epidermal cell counts were made within a determined leaf area of 0.68mm^2 for both leaf surfaces with a light microscope, Olympus - Vanox-T (Model AH2 - NAS, Tokyo, Japan), at X200 magnification. Stomatal frequencies (number of stomates per mm^2 of leaf surface) and stomatal indices [number of stomates / (number of stomates + number of epidermal cells) x 100 / unit area] were calculated from micrographs of three randomly selected fields per replicate of each leaf type.

The experiment was completely randomized. Data were analysed with an analysis of variance, and means were separated using Tukey's Multiple Range Test at 5% level of significance.

F. COMPARATIVE STUDY OF THE EPICUTICULAR WAX CONTENT ON LEAVES FROM TISSUE CULTURE-DERIVED AND CONVENTIONALLY PROPAGATED PLANTS

Leaf samples for wax extraction were taken from three different treatments. These included six-week old in vitro cultures on a solid hormone-free medium ready for transplantation, vigorously growing tissue culture-regenerated plants in the greenhouse that were nine-week old after transplantation, and conventionally propagated plants maintained in the greenhouse. The tissue culture-derived plants were transferred from culture directly to an open bench without any form of acclimatization. Two to three

fully expanded leaves per in vitro plant, as well as the youngest fully expanded leaf for each greenhouse plant were used in the experiment. The experiment was repeated three weeks later, using 9-week old in vitro cultures, 12-week old tissue culture-derived greenhouse plants, and the control non-micropropagated greenhouse plants.

Leaves were excised from each plant sample and held for 5-10 minutes at 20 $\pm$ 2°C and relative humidity of 35-40%. This was an attempt to ensure stomatal closure thus reducing the possibility of extraction of internal lipids. The sampled leaves were immersed into three consecutive dippings of 25-30ml chloroform contained in 250ml beakers. The extract was purified by filtering through anhydrous sodium sulfate placed on Pyrex wool in a glass funnel, into a glass container. A concentrated chloroform extract was obtained with a rotary evaporator (R-Bushi, Rinco Instrument Co.) and a water bath maintained at a temperature of 35-38°C in a fume hood. This was then poured into a pre-weighed aluminium pan, and left to evaporate in the fume hood before reweighing. The weight of the extracted epicuticular wax was obtained by computing the difference of the weighed aluminium pan before and after use and was expressed on a per unit area ($\mu g/cm^2$).

After wax extraction the area of the leaf sample was measured using a leaf area meter (Model PLI-3100 Lambda Instrument Corporation). Due to some fluctuations in the readings, the leaf area per sample was an average of three readings. A total of twelve replicates were used per treatment, and a

replicate set was completed before proceeding onto the next. Latex gloves were worn throughout the procedure to avoid contamination.

A completely randomized design was used in this experiment. Results were subjected to an analysis of variance, and the means were separated using Tukey's New Multiple Range Test at a probability of 5%. Statistical analyses were done with STATGRAPHICS and MSTAT-C computer packages.

CHAPTER 4

RESULTS AND DISCUSSION

PRELIMINARY OBSERVATIONS.

This study was initiated based on work by Acheampong and Henshaw (1984). These authors reported B5 salts supplemented with 5μM BAP and 0.05μM NAA were optimum for cocoyam shoot-tip initiation. Protocorm-like bodies formed in this medium within 18 weeks of culture, and subsequently produced plantlets only when transferred from agitated to a stationary liquid medium. They were successful in the propagation of cocoyam in vitro, but it required 18 weeks of culture. It did not report on earlier subcultures nor report factors that affect shoot and root production.

A preliminary study that compared shoot-tip initiation on Acheampong and Henshaw's medium (1984) with one that was growth regulator-free (Wutoh, 1989) showed better shoot development on the former medium. However, shoot proliferation, either through axillary or adventitious tissues, was absent even after 12 weeks of culture. Consequently, levels of 5 and 10μM BAP and 2μM TDZ were tested and subcultured at 4-6 weeks intervals in an attempt to obtain shoot proliferation. TDZ alone as well as in combination with BAP, enhanced axillary shoot proliferation. Elongation and proliferation of microshoots continued in media containing 5μM BAP alone,

and in combination with 0.05μM GA3. Microshoots at the level of 5μM BAP alone significantly outnumbered the shoots proliferated in the presence of GA3. GA3 at the level used was ineffective in promoting pronounced shoot elongation. A comparison of shoot lengths in both media showed 5μM BAP alone to produce a significant effect. Plantlets with well developed root systems were transplanted, and all survived the natural conditions in the greenhouse.

Efforts to regenerate cocoyam plantlets from callus and leaf tissues were not successful. Leaf disc cultures in various levels of 2,4-D, picloram, NAA and TDZ in MS basal salts and vitamins were evaluated in agitated liquid and solid media at low and high light intensities as well as in darkness. At low light intensities and darkness, cultures of picloram and NAA, both at 5, 10 and 25μM on solid and liquid media turned dark green and almost doubled the initial size after 2-4 weeks of culture. The cultures on solid media gradually turned yellow, thereafter, while liquid cultures stayed alive without further development.

Cocoyam shoot-tips were cultured in B5 and MS solid media in darkness according to Lui et al. (1988) and Hartman (1974), to induce callus formation. After two weeks of culture, the explant base in contact with media became swollen and callus formation was initiated in B5 medium, but no further development was observed with time.

These preliminary results lead to the further experiments described herein evaluating BAP and TDZ useage in the axillary branching procedure.

A. MEDIA SUBSTRATE INFLUENCE ON COCOYAM CULTURE

The summarized procedure for this experiment are shown in Figure 2.

1. Initiation of Culture

The disinfection procedure in this experiment resulted in low contamination, ranging from none in the solid medium to only 7% in agitated and stationary media. All explants survived as evidenced from their enlargement and manifested by an elongation of the shoot-tip and a swelling of the base by the second week of culture. At the same time, most cultures changed from the initial creamy color to a green coloration.

Initiation in the various substrates indicated that liquid culture, either on shaker or held stationary, was significantly better than filter paper bridges or solid medium (Table 1). Cultures on the latter two substrates developed 'tip dieback' by the fourth week and subsequently died. This was especially evident in 2μM TDZ where over 90% of the cultures showed this symptom (data not shown). Similar observations were apparent in the repeat of this experiment as well.

Preliminary evaluations and previous research indicated that 2μM TDZ and 5μM BAP are important in the initiation of cocoyam tissue cultures. A comparison of these two growth regulators (GR) with a high level of BAP (20μM) indicated that 2μM TDZ was significantly better with 5μM BAP intermediate and 20μM BAP least favorable (Table 2). The higher level of BAP (20μM) strongly inhibited shoot elongation.

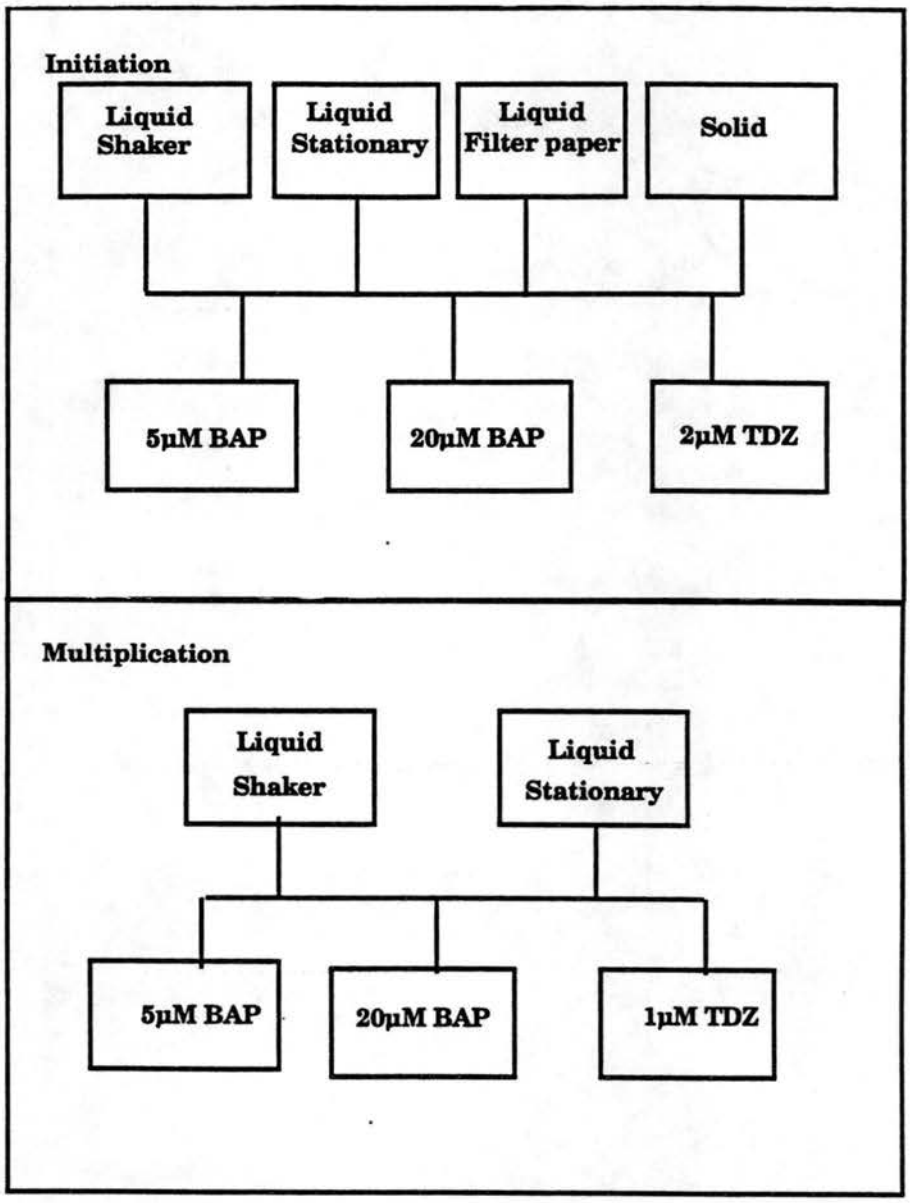


Figure 2. A summary of experiments testing media substrate influence on cocoyam culture.

Table 1. Effect of substrates on relative growth of cocoyam shoot-tips cultured on growth regulator treatments at 4 weeks from initiation.

Medium Substrate	Mean Rating*
Solid	1.8 b**
Liquid-filter	1.9 b
Liquid-shaker	2.4 a
Liquid-stationary	2.4 a

* Rating scale was as follows:
1) creamy or dead cultures showing no apparent growth,
2) initiation of growth and appearance of green coloration,
3) increase in growth, green coloration and leaf differentiation,
4) development of healthy green leaves.
** Values are averaged over three growth regulator treatments. Means followed by different letters within the column are significantly different at the 5% level with Tukey's Multiple Range Test.

Table 2. Effect of growth regulator levels on relative growth of cocoyam shoot-tips cultured on medium substrates at 4 weeks from initiation.

Growth Regulator Level	Mean Rating*
20μM BAP	1.6 c**
5μM BAP	2.2 b
2μM TDZ	2.7 a

* Rating scale was as follows:
1) creamy or dead cultures showing no apparent growth,
2) initiation of growth and appearance of green coloration,
3) increase in growth, green coloration and leaf differentiation,
4) development of healthy green leaves.
** Values are averaged over four substrates. Means followed by different letters within the column are significantly different at the 5% level with Tukey's Multiple Range Test.

An analysis of variance indicated that the interaction of GR and substrate was significant (Table 40, see Appendix). The level of 2μM TDZ in stationary culture was optimum for initiation of cocoyam shoot-tip cultures (Table 2).

2. Multiplication of Cocoyam

Cocoyam shoot-tips from the agitated and stationary liquid initiation experiments were subsequently evaluated on their respective substrates, at three GR levels of 1μM TDZ and at 5 and 20μM BAP. Significantly greater numbers of axillary shoots were observed on stationary liquid, 6.4, than on agitated liquid, 2.2 (Table 3). Significant differences were also observed among the GR treatments. The 1μM TDZ treatment gave rise to an average of 9.1 shoots per shoot tip as compared to 1.9 and 0.8 on 5 and 20μM BAP, respectively (Table 4). The significant interaction between media substrate and growth regulator level is shown in Table 41 (see Appendix).

These results agree with earlier findings that the choice of the physical state of a medium should not be made arbitrarily (Murashige, 1974). Initiation of cocoyam shoot tips on either a solid medium or stationary liquid with filter paper bridges was unsuccessful. Plant growth and proliferation was best on stationary liquid without any supports. Acheampong and Henshaw (1984) observed that agitated liquid media initiated the development of protocorm-like bodies, which differentiated into plantlets upon transfer into stationary liquid media. Thus, the state of the nutrient medium apparently played a significant role in determining the pattern of organogenesis in cocoyam. Jackson et al.

Table 3. Effect of substrates on numbers of shoots multiplied on growth regulator levels at 6 weeks from subculture.

Medium Substrate	Mean No. of Shoots*
Liquid-shaker**	2.2 b
Liquid-stationary	6.4 a

* Means followed by different letters within the column are significantly different at the 5% level with Tukey's Multiple Range Test.
** Cultures were initiated on liquid media on shaker and stationary, and these means are averaged from three growth regulator levels (1μM TDZ, 5 and 20μM BAP).

Table 4. Effect of growth regulator levels on numbers of cocoyam shoots multiplied on liquid substrates at 6 weeks from subculture.

Growth Regulator Level	Mean No. of Shoots*
20μM BAP**	0.8 c
5μM BAP	1.9 b
1μM TDZ	9.1 a

* Means followed by different letters within the column are significantly different at the 5% level with Tukey's Multiple Range Test.
** Cultures were initiated on agitated and stationary liquid substrates and these are the average from both substrates.

(1977b) also noted the poor growth of taro cultured on agar medium. Although it is a common practice to subject liquid nutrients to some form of agitation, agitated nutrients is not of absolute necessity with cocoyam (Table 1). Aeration, an important reason for agitation, was achieved in stationary liquid cultures due to floating of explants on the medium.

The observation that stationary liquid cultures are sufficient for initiation is valuable in that it simplifies the procedure. Costly agar and shakers, which may breakdown, are not needed. Time-consuming filter paper bridge construction is not needed as well. This is especially important in areas of the world where many media components have to be imported. Goodwin and Adisarwanto (1980), working with potato, eliminated the use of space-occupying flasks and expensive shakers with stationary liquid when they used stacked petri dishes.

B. THIDIAZURON INFLUENCE ON SHOOT-TIP INITIATION

A comparison of BAP at a level that was previously reported as optimum for cocoyam initiation (Acheampong and Henshaw, 1984) was made with TDZ. TDZ at 1, 2, 4 and 8μM were compared with 5μM BAP as the control using two explant sizes (Figure 3). By the second week of culture, a few shoots were formed in some of the cultures, but no significant differences were found among treatments nor at four and six weeks of observation (Table 5). Although the differences were not significant, 2μM TDZ produced the

Treatment	Explant Size (mm)	Growth Regulator
1	3-5 6-10	1μM TDZ
2	3-5 6-10	2μM TDZ
3	3-5 6-10	4μM TDZ
4	3-5 6-10	8μM TDZ
5	3-5 6-10	5μM BAP

Figure 3. A summary of experiments testing thidiazuron influence on shoot-tip initiation.

Table 5. Effect of growth regulator levels during culture initiation on number of cocoyam shoots with time in culture.

Growth Regulator Level	Weeks 2	4	6
5μM BAP	0.5 a*	0.7 a	1.2 a
1μM TDZ	0.6 a	1.1 a	1.5 a
2μM TDZ	0.4 a	1.3 a	2.0 a
4μM TDZ	0.5 a	1.1 a	1.3 a
8μM TDZ	0.8 a	1.3 a	1.5 a

* Means of 18 $\pm$ 2 replicates with those followed by the same letter in a column not significantly different at the 5% level with Tukey's Multiple Range Test.

greatest number of shoots after six weeks (Table 5). Its effect on shoot proliferation and growth (data not shown) apparently increased steadily over time.

Roots formed in the control treatment (5μM BAP) by the second week of culture, but none were evident in any of the TDZ treatments (data not shown). The suppression of root formation in TDZ treatments has been reported in other species (Kerns and Meyer, 1986; Lee and Wetzstein, 1990; Malik and Saxena, 1992).

The initial size of the explant affected its growth and development. Growth was assessed by shoot length and size of the base. Dissimilar effects of the GR levels were found from both explant sizes. The larger explants established and developed faster than smaller ones (Table 6). After two weeks of culture, larger explants were three-fold the size of the smaller ones, but only double that of the smaller explants by the end of six weeks. The GR levels showed no significant differences in effecting explant growth (data not shown). These results agree with earlier reports (George and Sherrington, 1984) which attributed differences to the contents of the cells. Okazawa et al. (1967) surmised that larger explants contained greater amounts of natural hormones than the smaller ones. The effect of explant size on the number of axillary shoots formed was significantly different at four and six weeks of culture (Table 7).

Table 6. Effect of explant size on growth (mm) of cocoyam shoots on initiation media with time in culture.

Size (mm)	Weeks 2	4	6
3 - 5	8.1 b*	13.5 b	22.4 b
6 - 10	28.1 a	38.0 a	40.7 a

* Means of 46 $\pm$ 1 replicates averaged over five growth regulator levels. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 7. Effect of explant size on cocoyam shoots initiated on growth regulator treatments with time in culture.

Size (mm)	Weeks 2	4	6
3 - 5	0.7 a*	0.7 b	1.0 b
6 - 10	0.4 a	1.4 a	2.0 a

* Means of 46 $\pm$ 1 replicates averaged over five growth regulator levels. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

C. INTERACTION OF TDZ AND BAP ON COCOYAM CULTURE

1. Initiation of Cultures on Agitated Liquid Media

Cultures initiated in liquid media on a shaker were well established by the second week. A contamination rate of 3.2% was observed. GR treatments included 0, 10 and 20μM BAP factorially combined with 0 and 2μM TDZ (Figure 4). The rate of shoot proliferation was slow in all six treatments during the six weeks of culture initiation. No significant differences were found for treatment effects on shoot proliferation (Table 8). Few shoots per culture formed, ranging from 0.4 for the cytokinin-free control to 1.7 for the treatment supplemented with 20μM BAP and 2μM TDZ combined. By the end of six weeks of culture, some shoot tips had differentiated into plantlets with well developed shoots and extensive root systems. Marked differences, significant at the 5% level, were observed relative to root initiation with the greatest number observed in the cytokinin-free control with a mean of 9.5 roots per culture. Although the lowest number of roots per culture were found in the combination of 20μM BAP and 2μM TDZ treatment (0.1), no significant differences were found among treatments which included cytokinins (Table 8). Obviously, the presence of TDZ and BAP repressed root growth.

1.1. Enhanced Axillary Shoot Multiplication

A comparison of 2μM TDZ with 20μM BAP in the multiplication media was made as well as their interaction with the effect of the initiation treatments on shoot growth and proliferation. The proliferation rate was low

Initiation

Treatments		1	2	3	4	5	6
Growth Regulator Levels (μM)	TDZ	2	2	2	0	0	0
	BAP	0	10	20	0	10	20

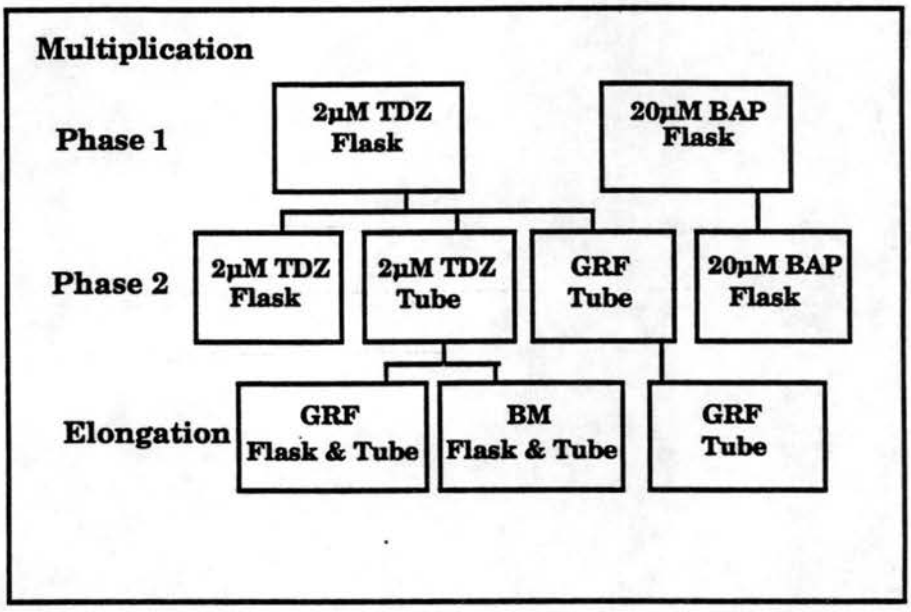


Figure 4. A summary of experiments testing agitated liquid media on cocoyam cultures.

Table 8. Effect of cytokinins on shoot proliferation and root formation of cocoyam shoot tips after 6 weeks in initiation media.

Cytokinin (μM) BAP	TDZ	Culture No.	Shoot No.	Root No.
0	2	21	1.6 a*	0.2 b
10	2	21	1.6 a	0.3 b
20	2	21	1.7 a	0.1 b
0	0	19	0.4 a	9.6 a
10	0	20	1.1 a	1.1 b
20	0	20	1.4 a	0.1 b

* Means with the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

in all the treatments for the first two weeks in the multiplication media, but increased substantially by the fourth week (Table 9). Shoot formation originated from the axillary meristems at the nodal points, found just above the cut base end. Further shoot formation occurred at the leaf axils of both the principal shoot and developing microshoots (Figure 5). The first shoots produced appeared to have developed from existing axillary buds, that were present at excision, which were subsequently freed from apical dominance by exogenous GRs. The leaves were initially cordate with acute tips, and became more sagitate as they developed. Shoot formation was direct without the formation of callus as reported in the work by Hartman (1974), or protocorm-like bodies as reported by Acheampong and Henshaw (1984). However, a few cultures irregardless of growth regulator treatment had protocorms.

The effect of TDZ and BAP on axillary shoot proliferation was similar after four weeks of culture, 8.6 and 7.4 respectively. However, the difference was significant at six weeks of culture, where the TDZ effect (13.1) was superior to that of BAP (9.7, Table 9). These findings are similar to those reported on grape by Sudarsono and Goldy (1988), but contrary to that of Gray and Benton (1991), who also worked with grape. These results indicated that TDZ was more effective than BAP in breaking apical dominance and enhancing axillary shoot formation. TDZ produced more compressed shoots, while BAP cultures produced shoots that more easily differentiated into well-defined plantlets with eventual formation of extensive root systems. There were no significant differences in shoot length at four weeks of culture,

Table 9. Effect of cytokinins in multiplication media on cocoyam shoot proliferation, root formation and shoot length at 4 and 6 weeks.

Cytokinin (μM)	Shoot No.* 4	6	Root No. 4	6	Shoot Length(mm)** 4	6
BAP (20)	7.4a	9.7b	2.0a	6.0a	55.2a	75.0a
TDZ (2)	8.6a	13.1a	1.4a	1.7b	60.4a	65.1b

* Each value is a mean of 60 replicates averaged over six initiation growth regulator treatments. Means followed by the same letter in a column are not significantly different at the 5% level according to Tukey's Multiple Range Test.

** Shoot length represents length of the main shoot.

Figure 5. Proliferated axillary shoots of cocoyam at 6 weeks in multiplication medium.

but BAP showed greater length at six weeks. Similar findings were reported with muscadine grape (Gray and Benton, 1991) where TDZ resulted in stunted shoots as compared to BAP.

The development of roots was similar at four weeks of culture, but showed significant differences at six weeks (Table 9). BAP allowed root formation, while TDZ did not. Cultures with BAP produced basal roots which were branched and had discernible root hairs. Rooting in BAP may have been enhanced because of the NAA incorporated into the media. In muscadine grape, rooting was impossible in the presence of BAP with higher levels affecting subsequent rooting when transferred on media without BAP (Lee and Wetzstein, 1990). Evidently in cocoyam the 0.05μM NAA was sufficient to induce root formation in the presence of BAP, even at a level of 20μM, but not with TDZ. There have been other reports where TDZ repressed root formation (Gray and Benton, 1991; Malik and Saxena, 1992) as well.

An analysis of the influence of the initiation treatment during the multiplication phase indicated that there were differences associated with the GRs used in the initiation of the cultures. The average number of shoots per culture ranged from 3 in the cytokinin-free control to 10.9 in treatments with 20μM BAP as well as in the 20μM BAP and 2μM TDZ combined after 4 weeks of culture (Table 10). After 6 weeks, it ranged from 4.3 in the control to 16.2 in 2μM TDZ. Although the means appear low, shoot numbers of 34-37 were obtained in some cultures. Axillary shoot proliferation was markedly increased between the fourth and sixth weeks. It is apparent from a comparison of the

Table 10. Effect of initiation treatments on cocoyam shoot proliferation, root formation and shoot length during multiplication on 2μM TDZ and 20μM BAP.

Growth Regulator (μM)		Shoot No.*		Root No.*		Shoot Length (mm)*	
BAP	TDZ	4**	6	4	6	4	6
0	2	10.9a	16.2a	0.0c	0.9c	55.8a	71.4a
10	2	6.9ab	10.8ab	0.3c	2.0bc	62.4a	75.8a
20	2	9.1b	12.7a	0.2c	1.7c	39.3a	61.1a
0	0	3.0b	4.3b	4.4a	7.7a	54.8a	65.2a
10	0	7.2ab	9.8b	3.1ab	5.8a	66.1a	79.3a
20	0	10.1a	14.6a	2.2bc	5.0ab	58.6a	67.6a

* Values are the average of 20 replicates of 2μM TDZ or 20μM BAP. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

** Weeks after subculture.

effects of the GR levels in multiplication media to those in the previous initiation phase that the influence of BAP on shoot proliferation tends to diminish while that of TDZ increases with time in culture. This is apparent in Figure 6, where the effects of BAP and TDZ on axillary shoot proliferation is illustrated as a function of culture age, during multiplication. TDZ continued to effect a marked increase even after 8 weeks in culture, while shoot proliferation in BAP gradually levelled off (Figure 6). Although there were significant effects, shoots produced in BAP appeared more normal and developed faster than those produced in TDZ, most of which were small and stunted.

The initial GR levels showed no significant differences on shoot length at 4 and 6 weeks of culture, (Table 10). However, the smallest shoots were observed in the control treatment and that containing 20μM BAP and 2μM TDZ. The elevated levels of cytokinin apparently had some limiting effect on shoot growth. Similar findings have been reported where higher BAP levels strongly inhibited shoot elongation of muscadine grape (Lee and Wetzstein, 1990).

1.1.1. Cocoyam Proliferation upon Subculture into Multiplication and Growth Regulator-free Media.

When shoots were recultured into their respective fresh media, the effect of TDZ on axillary shoot formation was again apparent with significantly greater proliferation rates than that of BAP (Table 11). There was no marked increase in shoot numbers between four and six weeks, thus indicating that

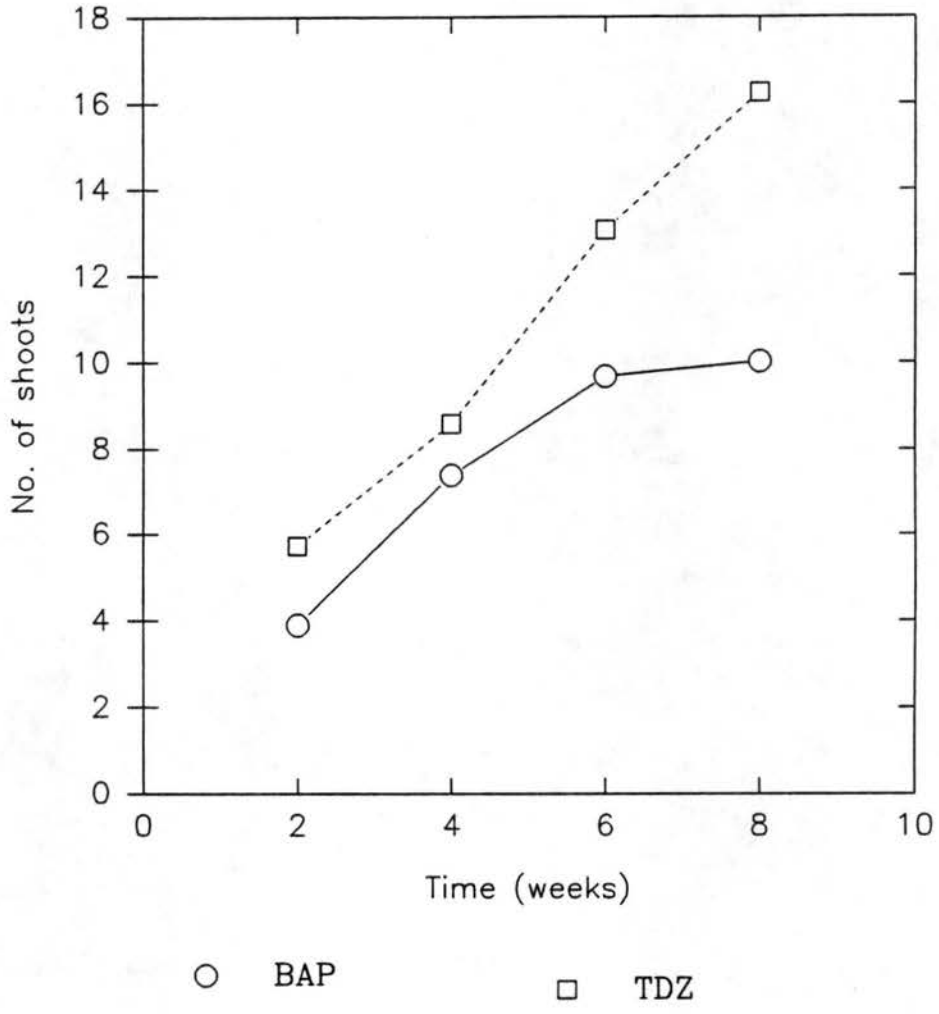


Figure 6. **Effect of BAP and TDZ in multiplication media on axillary shoot proliferation with time.**

Table 11. Effect of cytokinin treatments during multiplication on subsequent cocoyam shoot and root proliferation.*

Cytokinin (μM)	Shoot No.**		Root No.**	
	4***	6	4***	6
BAP (20)	14.5 b	15.6 b	7.1 a	9.8 a
TDZ (2)	31.3 a	32.9 a	0.0 b	0.0 b

* Cultures were initiated on six media and subcultured once on 20μM BAP or 2μM TDZ.

** Values are means of 58 $\pm$ 1 replicates. Means followed by a different letter within a column are significantly different at the 5% level with Tukey's Multiple Range Test.

*** Weeks after subculture.

subcultures could be made directly from four week old cultures. Cultures tended to respond more to the effect of the growth regulator in the multiplication medium than to the initial one.

Initiation treatments effected shoot and root proliferation similarly during multiplication phase, when 2μM TDZ and 20μM BAP were used in the multiplication media. Mean number of axillary shoots ranged from 18.5 for 20μM BAP to 26.7 for the cytokinin-free control after four weeks, and 19.7 for 20μM BAP to 28.1 for the control after six weeks of culture (Table 12). The absence of significance among initiation treatments may not have implied that treatment effect on shoot multiplication was equal, since some variability was observed within treatments. A level of 20μM BAP in the multiplication phase greatly affected shoot formation. Cultures initiated in 20μM BAP as well as 10μM BAP and 2μM TDZ produced the least number of shoots although not significant. This is similar to the results from the earlier experiment in that an initiation with 20μM BAP represses shoot growth, and thus proliferation. When these repressed tissues are further maintained in the same medium, their proliferation is restricted. Lee and Wetzstein (1990) observed high mortality of muscadine grape shoots at 20μM BAP and higher levels. In this study, TDZ had a tendency to enhance the initial BAP effects.

Shoot proliferation and root formation were negatively correlated. The superiority of BAP to TDZ on root formation is once again evident (Table 11). Shoots proliferated on BAP developed roots by the second week in culture. The roots were elongated, highly branched and produced numerous root hairs.

Table 12. Effect of initiation treatments, after subculturing twice, on subsequent cocoyam shoot and root proliferation.*

Growth Regulator (μM)		Shoot No.**		Root No.**	
BAP	TDZ	4***	6	4	6
0	2	25.2 a	25.8 a	3.4 a	4.9 a
10	2	19.9 a	21.1 a	3.4 a	4.3 a
20	2	23.4 a	26.3 a	2.7 a	4.3 a
0	0	26.7 a	28.1 a	3.8 a	5.0 a
10	0	25.7 a	26.3 a	2.3 a	3.7 a
20	0	18.5 a	19.7 a	4.7 a	6.0 a

* Cultures were subcultured once on the same growth regulator treatment of 20μM BAP or 2μM TDZ.

** Values are the average of 18 $\pm$ 1 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

*** Weeks after subculture.

The poor rooting ability in TDZ as compared to the previous multiplication phase indicates that as long as the cultures are on TDZ, the greater its repressive effect on rooting. The effect of the individual initial treatments was not significant at either time of observation. Root number ranged from 2.3 and 3.7 for 10μM BAP to 4.7 and 6.0 for 20μM BAP, at four and six weeks of culture, respectively (Table 12).

Shoots multiplied on 2μM TDZ were used in subsequent proliferation and rooting evaluations. These shoots were numerous and rootless (Table 11). Results of subcultures made into 2μM TDZ and growth regulator-free (GRF) agar-solidified media did show marked differences. Axillary shoot proliferation in 2μM TDZ was significantly greater as compared to the growth regulator-free medium (Table 13). Shoots continued to proliferate without growth regulators, but the effect of 2μM TDZ doubled and almost tripled that of the former after four and six weeks of culture, respectively, (Table 13). The 2μM TDZ medium increased shoot proliferation from four to six weeks of culture. Shoot proliferation in GRF medium probably reached a maximum at four weeks. It may be best to reculture into a fresh medium at four weeks to optimize proliferation. Continued proliferation of shoots in a medium without GRs may be the result of the cumulative effect of GRs in previous media.

A comparison of root formation in 2μM TDZ and GRF multiplication media substantiates the inhibition of rhizogenesis by TDZ, while all GRF cultures rooted. Shoots in GRF medium readily produced elongated roots with profuse root hairs and secondary roots, even though cultures were previously

Table 13. Effect of 2μM TDZ and growth regulator-free solid multiplication media on shoot and root proliferation.*

Growth Regulator Level	Shoot No.**		Root No.**	
	4***	6	4***	6
2μM TDZ	27.6 a	34.1 a	0.0 b	0.0 b
None	13.7 b	13.5 b	8.0 a	15.5 a

* Cultures had been initiated on various growth regulator combinations but were subsequently subcultured once on 2μM TDZ before subcultured onto above media.

** Values are means of 59 $\pm$ 1 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

*** Weeks after subculturing.

in a TDZ (root-inhibiting) medium (Table 13). Kerns and Meyer (1986) reported similar results for *Acer* x *freemanii* cultures. Although the mode of action of TDZ in organogenesis is yet to be determined, it is possible that root induction is initiated in its presence, but development is delayed, and expressed in its absence.

The interaction of the initiation GRs with multiplication media was found significant (Table 42, see Appendix). An examination of the individual treatments supports the observation that TDZ effect was superior to that of GRF. However, there was a tendency of more shoots to be produced in the GRF medium from 2μM TDZ and 20μM BAP combined, as well as the cytokinin-free initiation level as compared to others (Table 14). The joint effect of 2μM TDZ and 20μM BAP initiation treatment on 2μM TDZ multiplication medium almost doubled that produced in GRF medium, whereas comparable effects were found for the cytokinin-free control on both 0 and 2μM TDZ media. Proliferating shoots of the cytokinin-free control and the joint effect of 2μM TDZ and 20μM BAP initiation treatments multiplied on 2μM TDZ is illustrated on Figure 7.

Shoot proliferation in solid and agitated liquid 2μM TDZ media was not significant (Table 15). An increase was observed in solid medium between the two periods of observation. Hence, shoots can be proliferated in either solid or liquid media, with a tendency of getting larger numbers in solid media. The present findings are in contrast to a previous report (Ng and Hahn, 1985)

Table 14. Effect of initiation growth regulator treatments and the presence or absence of 2μM TDZ during multiplication on subsequent shoot proliferation.

Growth Regulator Level (μM)			Shoot No.*	
Initiation		Multiplication	4**	6
BAP	TDZ	TDZ		
0	2	2	35.1 a	45.3 a
10	2	2	31.1 ab	38.8 ab
20	2	2	30.0 ab	32.7 abc
0	0	2	18.4 abc	24.4 bcde
10	0	2	26.9 abc	32.2 abcd
20	0	2	24.7 abc	32.1 abcd
0	2	0	13.8 bc	13.9 de
10	2	0	9.6 c	9.3 e
20	2	0	16.9 bc	17.3 cde
0	0	0	18.7 abc	18.0 cde
10	0	0	10.4 c	9.2 e
20	0	0	12.6 c	12.6 e

* Values are means of 10 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

** Weeks after subculture. Cultures had been subcultured once on 2μM TDZ.

A

B

Figure 7. Proliferating cocoyam cultures on 2μM TDZ at 3 weeks.
A. Culture had been initiated on basal medium without cytokinin.
B. Culture had been initiated on 2μM TDZ and 20μM BAP.

Table 15. Effect of medium substrates during multiplication phase (2μM TDZ) on shoot proliferation.

Substrate	Shoot No.*	
	4**	6
Liquid-shaker	31.3 a	32.7 a
Solid	27.6 a	34.1 a

* Values are means of 58 $\pm$ 2 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

** Weeks after subculture. Cultures were initially derived from various growth regulator combinations and subcultured twice on 2μM TDZ.

that did not find cocoyam plantlet formation in agitated liquid cultures. Such a discrepancy could be cultivar related.

1.1.2. Effect of Basal and Growth Regulator-free Media on Cocoyam Proliferation

To further investigate the influence of no GR medium on shoot proliferation, a comparison was made with BM that contained 0.05μM NAA. Microshoots obtained from 2μM TDZ cultures were harvested and subcultured into these two media for a period of 4 weeks. There were no significant differences between the effect of BM and GRF media on the number of shoots and roots formed (Table 16). NAA may have been unnecessary at this point to sustain shoot formation and induce rooting of microshoots. The efficiency of morphogenesis in GRF media is probably due to either of two reasons: expression from endogenous hormones that may have been activated by the exogenous GRs, or a carry-over effect of GRs. There was no significant interaction between the initiation GR levels with the multiplication-elongation media.

The initial growth regulator levels produced significant differences on subsequent shoot proliferation. An initiation level of 2μM TDZ resulted in the lowest proliferation rate (23.7/culture). It was significantly different from the effect of 20μM BAP (38.3) as well as with the combined effect of 2μM TDZ and 20μM BAP (37.6) (Table 17).

Average number of roots formed were 10.5 and 9.5 for GRF and BM media, respectively (Table 16). The differences in root formation for different

Table 16. Effect of the presence of growth regulator in elongation media on cocoyam shoot and root proliferation at 4 weeks of subculture.*

Growth Regulator	Shoot No.**	Root No.**
None	31.8 a	10.5 a
NAA (0.05μM)	31.4 a	9.5 a

* Cultures were initiated on various growth regulator combinations and subcultured twice on 2μM TDZ before subculturing in above media.
** Each value is an average of 72 replicates. Means followed by the same letter within a column are not significantly different at the 5% level of significance according to Tukey's Multiple Range Test.

Table 17. Effect of growth regulator levels at the initiation stage on subsequent cocoyam shoot and root proliferation on growth regulator-free and basal media at 4 weeks of culture.*

Growth Regulator (μM)		Shoot No.**	Root No.**
BAP	TDZ		
0	2	23.7 b	7.9 bc
10	2	28.7 ab	15.4 a
20	2	37.6 a	7.6 bc
0	0	30.8 ab	10.5 abc
10	0	30.4 ab	13.3 ab
20	0	38.3 a	5.5 c

* Cultures were subcultured twice on 2μM TDZ then into growth regulator-free (GRF) and basal media (BM). No significant interaction was found between initial growth regulator treatments and GRF and BM.
** Values are means of 24 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

initiation GRs were significant in some cases (Table 17). The least number of roots were formed in media initiated in higher cytokinin concentrations, that is, 2μM TDZ and 20μM BAP, individually and in combination. This substantiates the effect of the previous GRs on subsequent rooting.

1.1.3. Effect of Culture Vessel on Cocoyam Proliferation

In a study of the effect of culture vessels on organogenesis, shoot clusters harvested from 2μM TDZ cultures were subcultured in GRF medium either in test tubes or flasks. No significant effect was found between vessels on shoot proliferation and root formation (Table 18). Roots were formed in all cultures, but the average of 10.5 roots per culture in test tube and 12.0 roots per culture in flask was small when compared with the number of shoots formed. Roots were formed only by the older more developed shoots. Flask cultures had well expanded leaves that could be attributed to their larger culture environment. However, the high proliferation rates in both containers indicate that either of them may be used. It should be noted that the use of flasks was labor intensive as it required cotton plugs and aluminium covers, unlike test tubes which could be easily capped. Furthermore, flasks took up considerably more space.

The effect of the initiation GR levels on shoot formation in flasks and tubes (Table 19) is similar to the response obtained in the comparison of GRF and BM media (Table 17). Significant differences were found among the effects of 20μM BAP (34.7), 2μM TDZ combined with 20μM BAP (39.4), and that of 2μM TDZ (20.7) (Table 19). Although the precise mode of action of

Table 18. Effect of culture vessels on cocoyam shoot and root proliferation on growth regulator-free medium after 4 weeks from subculture.*

Culture Vessel	Shoot No.**	Root No.**
Flasks	30.2 a	12.0 a
Test Tube	31.8 a	10.5 a

* Cultures were subcultured twice on 2μM TDZ, and then transferred into growth regulator-free medium in the above culture vessels.
** Values are means of 72 replicates of six initial growth regulator combinations. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 19. Effect of initiation growth regulator treatments on subsequent cocoyam shoot and root proliferation in growth regulator-free medium after 4 weeks from culture.*

Growth Regulator (μM) BAP	TDZ	Shoot No.**	Root No.**
0	2	20.7 b	13.0 a
10	2	29.3 ab	11.8 a
20	2	39.4 a	5.4 b
0	0	29.6 ab	13.3 a
10	0	32.1 ab	14.8 a
20	0	34.7 a	9.2 ab

* Plantlets had been subcultured twice in 2μM TDZ before being transferred into growth regulator-free medium in flasks and tubes.
** Values are means of 24 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

TDZ is still to be elucidated, these results indicate that growth on TDZ drastically reduces subsequent shoot proliferation in cultures when transferred to GRF and BM media but is enhanced in the presence of BAP. The least number of roots were found in 2μM TDZ combined with 20μM BAP (5.4) which were significantly less than the other treatments.

1.1.4. Effect of Continuous Use of Growth Regulator-Free Media on Cocoyam Proliferation

Shoots continuously subcultured in a GRF medium show that shoot proliferation can continue in a medium lacking GRs. A comparison of shoot numbers effected by initiation GR treatments was not significantly different. The average number of shoots per culture ranged from 7.4 and 8.1 for 10μM BAP to 12.1 and 13.2 for 2μM TDZ combined with 20μM BAP, at two and four weeks of culture, respectively (Table 20). When compared to the number of shoots formed in the previous GRF medium (see Table 14), it was apparent that shoot production diminished as cultures were maintained without GRs. The average root number per culture ranged from 2.4 for the combined effect of 2μM TDZ and 20μM BAP to 6.1 for 20μM BAP after 2 weeks of culture (Table 20). More roots formed at the end of 4 weeks, when the average number of roots increased to 11.6 for 2μM TDZ to 15.3 for the combined action of 2μM TDZ and 20μM BAP, but still with no significant differences among treatments.

Due to the ease of rooting cocoyam shoots in the presence of BAP, as well as during elongation in a GRF medium, no specific studies were focused

Table 20. Effect of initiation growth regulator levels on subsequent cocoyam shoot and root proliferation after 2 and 4 weeks of culture into growth regulator-free medium.*

Hormone Level (μM)		Shoot No.**		Root No.**	
BAP	TDZ	2	4	2	4
0	2	9.1 a	11.1 a	5.8 a	11.6 a
10	2	9.3 a	10.4 a	4.9 a	15.2 a
20	2	12.1 a	13.2 a	2.4 a	15.3 a
0	0	9.7 a	12.1 a	4.9 a	14.3 a
10	0	7.4 a	8.1 a	4.6 a	13.3 a
20	0	8.6 a	9.1 a	6.1 a	13.6 a

* Plantlets had been cultured into 2μM TDZ followed by an initial culture in growth regulator-free (GRF) medium prior to subculturing into GRF medium.
** Values are means of 14 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

on rooting. The type of explant material transferred into the GRF medium determined the morphogenesis. Shoot proliferation like root formation, continued in GRF medium when shoot clusters served as explants. However, if single shoots of less than one-half centimeter were isolated, elongation and root formation occurred with limited or no proliferation (Figure 8). Single shoots elongated more rapidly than clustered shoots, bearing three to four leaves and approximately seven roots per plantlet (data not shown). These plantlets, approximately 4cm in height, were ready for transplantation to the greenhouse by the third week of culture.

2. Initiation of Cultures on Stationary Liquid Media in Test Tubes

Shoot tips from plants established in the greenhouse from in vitro-cultured plantlets were explant source for a study on the interaction of BAP at 5 and 10μM factorially combined with TDZ at 0, 1, 2 and 4μM, and a control treatment lacking cytokinins. The experimental procedures are illustrated in Figure 9. After six weeks of initiation in stationary liquid, the results were similar to those of other experiments where few shoots developed and no significant differences were observed due to treatment effect. The average number of new shoots ranged from 0.1 for the joint effect of 10μM BAP and 1μM TDZ to 0.7 for the cytokinin-free control. These results seem to indicate that shoot proliferation in cocoyam is time-dependent, and that culture initiation is principally for explant establishment. Roots formed in media without TDZ. The highest average root number was produced in the control (9.6), with 100% rooting. Its effect was significantly greater

Figure 8. Cocoyam plantlet proliferated from axillary shoots.

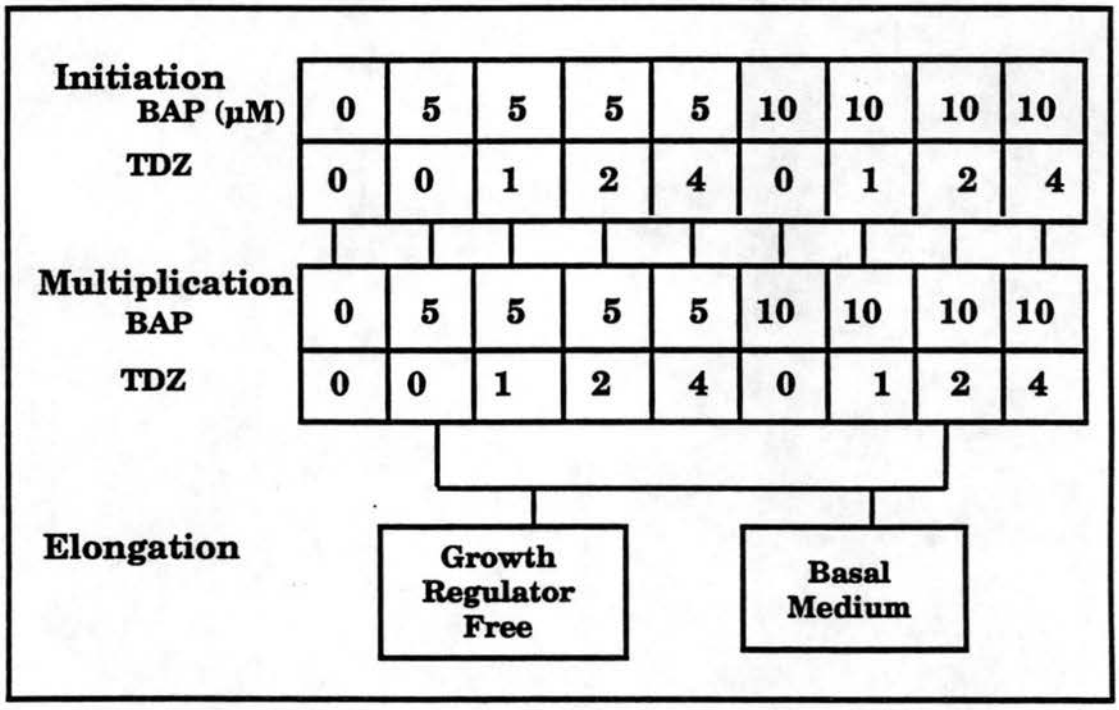


Figure 9. A summary of experiments testing the influence of stationary liquid media in test tubes on cocoyam cultures.

than the others. Root frequencies of 100%, 53% and 6.67% occurred in control, 5μM and 10μM BAP, respectively.

2.1. Enhanced Axillary Shoot Multiplication

Explants from the initiation media were transferred to fresh media with the same growth regulators for multiplication. Axillary shoots proliferated in the media by the fourth week of culture, showing an increase at six weeks (Table 21). The responses of the cytokinin-free control (0.5 shoot/culture) and 5μM BAP (6.7 shoots/culture) were significantly different at four weeks. However, the pattern of shoot production changed at six weeks, when the greatest number of shoots were produced in 10μM BAP combined with 1μM TDZ, an effect that was significantly different from that of the control but similar to other levels. There was an approximate doubling of shoot numbers from four to six weeks in all combinations of TDZ with BAP. The number of shoots that rooted at four and six weeks of culture ranged from 33.3% for 10μM BAP to 100% for the control and 5μM BAP (data not shown).

Growth regulators levels significantly affected rooting, and was the same for both 4 and 6 weeks (Table 21). Virtually no roots were formed in treatments containing TDZ, again emphasizing its suppressive effect on rooting.

2.2. Effect of Basal and Growth Regulator-Free Media on Cocoyam Proliferation

Shoot proliferation continued in GRF and cytokinin-free media (BM) (Table 22), as in an earlier experiment (Table 16). No significant differences

Table 21. Effect of growth regulator levels on cocoyam shoot proliferation and root formation at 4 and 6 weeks.*

Growth Regulator (μM)		Shoot No.**		Root No.**	
BAP	TDZ	4	6	4	6
0	0	0.5 b	0.8 b	11.6 a	12.9 a
5	0	6.7 a	6.7 ab	9.1 a	10.6 a
5	1	5.5 ab	8.4 ab	0.0 c	0.0 c
5	2	4.1 ab	9.1 ab	0.0 c	0.0 c
5	4	3.3 ab	6.7 ab	0.0 c	0.0 c
10	0	3.9 ab	4.8 ab	3.3 b	5.9 b
10	1	5.5 ab	10.0 a	0.0 c	0.0 c
10	2	2.2 ab	3.8 ab	0.0 c	0.0 c
10	4	3.9 ab	6.6 ab	0.0 c	0.0 c

* Cultures were initiated on the same media as they were subcultured onto for multiplication.
** Values are means of 15 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 22. Effect of absence or presence of growth regulator on cocoyam shoot and root formation after 4 weeks of subculture.*

Growth Regulator	Shoot No.**	Root No.**
None	20.4 a	10.7 a
NAA (0.05μM)	15.9 a	9.1 a

* Shoot tips were initiated and subcultured onto the same initiation medium prior to subculture onto the above elongation media. There was a significant interaction between initiation and elongation media.
** Values are pooled means of 115 replicates from the initiation treatments. Means followed by the same letter in a column are significantly different at the 5% level of significance with Tukey's Multiple Range Test.

were found between the GRF and BM media. There was a tendency for greater proliferation in GRF than in the BM medium, but the responses were not significant (Table 22). The percentage of shoots that rooted were 88.13% and 87.08% in GRF and BM media, respectively. The observation of morphogenesis in GRF is important because of the potential savings in routine propagation.

An examination of initial growth regulator levels showed that treatments with higher TDZ levels more efficiently enhanced proliferation, irrespective of the BAP levels (Table 23). This confirms earlier findings that TDZ is more efficient than BAP with time in promoting cocoyam shoot proliferation. The average shoot number produced per culture ranged from 5 for 5μM BAP to 31.7 for the combined effect of 5μM BAP and 4μM TDZ. It is evident from these results that a combined BAP-TDZ treatment is largely due to the effect of TDZ. The significant interaction between the multiplication and the GRF media suggests that the proliferation in GRF media could be a result of the carry-over effects from the multiplication media (Table 43, see Appendix). The opposite was observed for root formation. The highest rooting effect was found with the lowest TDZ levels that produced fewer shoots (Table 23).

3. Initiation of Cultures on Stationary Liquid Media in Erlenmeyer Flasks

3.1. Enhanced Axillary Shoot Formation

The summarized experimental procedures are shown in Figure 10. Shoot tips initiated in BM supplemented with 5μM BAP survived and increased

Table 23. Cocoyam shoot and root formation during elongation phase as influenced by growth regulator levels from multiplication media at 4 weeks.*

Growth Regulator (μM) BAP	TDZ	Shoot No.**	Root No.**
5	0	5.0 c	12.4 ab
5	1	9.7 c	15.2 a
5	2	20.3 b	5.0 c
5	4	31.7 a	5.6 c
10	0	5.4 c	13.1 ab
10	1	20.0 b	14.1 a
10	2	23.7 ab	9.1 bc
10	4	30.8 a	5.6 c

* Shoot tips were initiated on and subcultured onto the same growth regulator treatments prior to transfer to growth regulator-free media.

** Values are means of 25 $\pm$ 5 replicates. Means followed by the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

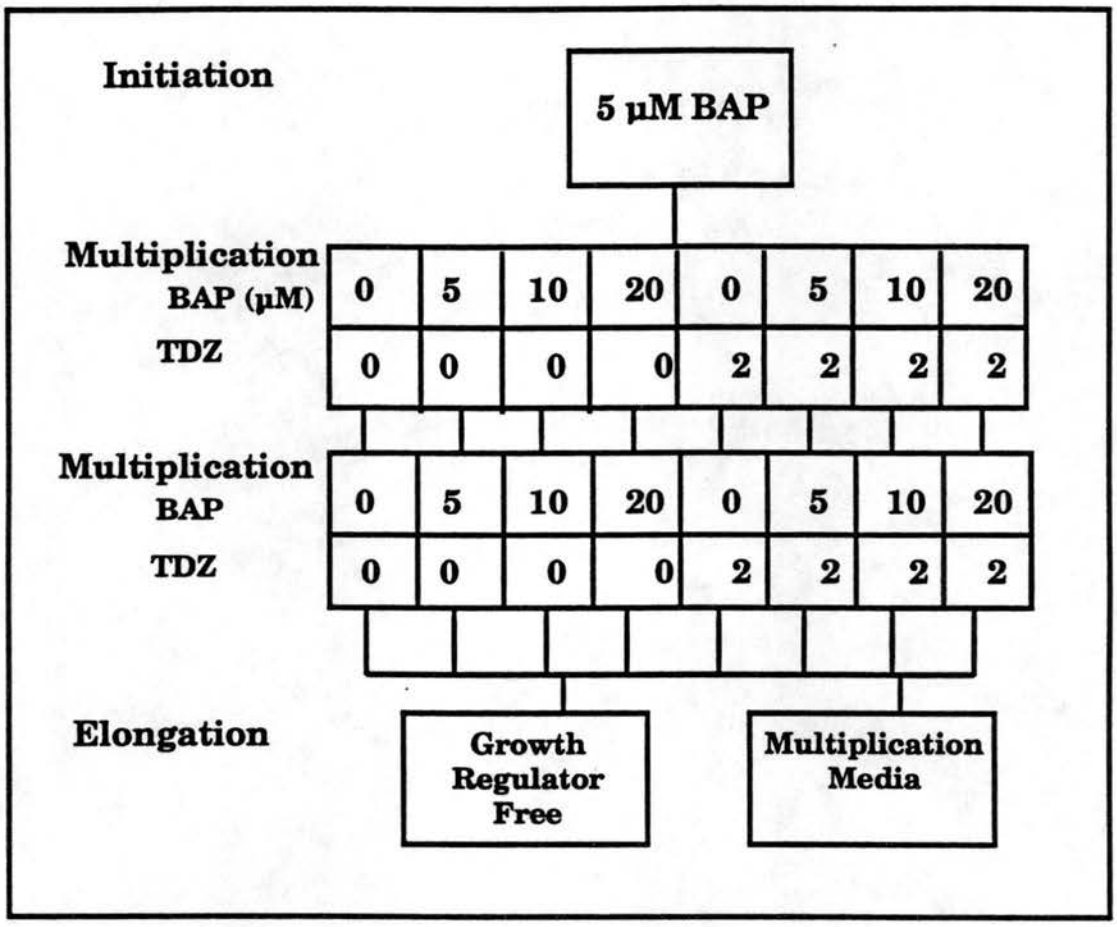


Figure 10. A summary of experiments testing stationary liquid media in Erlenmeyer flasks influence on initiation of cocoyam shoot-tip cultures.

in size. When transferred into multiplication media containing BAP at levels of 0, 5, 10 and 20μM factorially combined with TDZ at 0 and 2μM, shoot proliferation was low after six weeks of culture. The highest number of shoots produced resulted from treatments containing 2μM TDZ, with an average of 4.8 shoots per culture (Table 24). No shoots were produced from the cytokinin-free control (BM), and very little from treatments containing solely BAP. The low rate of shoot proliferation at this initial multiplication stage is indicative that the 4-week exposure in 5μM BAP was not helpful, and may have been unnecessary. The length of the main shoots was not significantly different after 4 weeks of culture, but significant differences were evident at 6 weeks (Table 24). Roots formed in all treatments, but significant numbers were found in BM and 5μM BAP (Table 24). Rooting of cultures in media containing TDZ was minimal as in previous experiments. The repressive effect of TDZ is again apparent in this experiment from the significantly reduced number of roots. The few cultures that rooted may have been the result of an interaction with the effect of BAP in the initiation medium. No significant correlation was found between the base diameter and the number of shoots proliferated (data not shown).

Shoots proliferated profusely when these explants were subcultured onto identical media (Table 25). After 4 weeks of subculture, an average of 36.6 shoots per culture were formed in the treatment containing both 20μM BAP and 2μM TDZ, as opposed to none in BM and only 7.1 in 5μM BAP. Shoot proliferation increased two weeks later in the same order, where an

Table 24. Effect of growth regulator levels on cocoyam shoot and root proliferation after 6 weeks in culture following initiation on 5μM BAP for 4 weeks.

Growth Regulator Level (μM) BAP	TDZ	Shoot No.*	Root No.*	Shoot Length*
0	0	0.0 c	8.9 a	61.3 a
5	0	0.5 bc	8.8 a	57.1 ab
10	0	0.8 bc	3.3 b	52.4 abc
20	0	1.7 abc	1.9 b	43.3 abc
0	2	4.8 a	0.6 b	33.6 bc
5	2	3.5 ab	0.1 b	47.4 abc
10	2	3.3 ab	0.3 b	32.0 c
20	2	3.2 abc	0.1 b	33.8 bc

* Values are means of 12 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 25. Effect of growth regulator levels on cocoyam shoot and root proliferation when recultured in multiplication media for 4 and 6 weeks.*

Growth Regulator Level (μM)		Shoot No.**		Root No.**	
BAP	TDZ	4	6	4	6
0	0	0.0 c	0.0 d	11.0 a	13.3 a
5	0	7.1 c	7.2 cd	9.1 a	12.2 a
10	0	10.8 bc	12.8bcd	3.6 bc	7.9 a
20	0	16.9 bc	23.1abcd	4.5 b	8.7 a
5	2	25.3 ab	30.3 ab	0.0 c	0.0 b
10	2	20.6 abc	30.8 bc	0.0 c	0.5 b
20	2	36.6 a	44.5 a	0.1 c	0.1 b

* Shoot tips were initiated on 5μM BAP and subcultured once on various growth regulator multiplication media prior to subculture onto the same multiplication media.

** Values are means of 10 $\pm$ 1 replicates. Means followed by the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

average of 44.5 shoots were produced from the combined effect of 20μM BAP and 2μM TDZ, none in BM and essentially no increase in 5μM BAP which had only 7.2 shoots. The treatment with 2μM TDZ was eliminated from the analysis due to insufficient data resulting from contamination. However, its effect at the initial multiplication phase, as well as results obtained in the other experiments, substantiates its shoot-enhancing ability.

The level of 0.05μM NAA in the BM did not effect apical dominance to allow growth of axillary shoots. Shoots in the 0.05μM NAA elongated rapidly and adventitious root formation was profuse, producing branched roots and numerous root hairs. At 4 weeks of subculture, an average of 11 roots were formed per culture, and 9.1 roots in 5μM BAP (Table 25). After 6 weeks, the average increased to 13.3 and 12.2 for BM and 5μM BAP, respectively. The effects of these treatments were significantly different from the others. The suppressive effect of TDZ on root formation was again apparent.

3.2. Effect of Multiplication and Growth Regulator-Free Media on Cocoyam Proliferation

A high level of contamination was observed when microshoots were subcultured from multiplication media into the same or GRF media. Therefore there was insufficient data for analysis of some treatments. However, the comparison was made with treatments containing 2μM TDZ, and 2μM TDZ combined with 20μM BAP. At 4 weeks of culture, 2μM TDZ produced an average of 17.5 shoots per culture, while 20μM BAP and 2μM TDZ combined had 9.8 shoots (Table 26). Shoots proliferated in the GRF medium were

Table 26. Effect of growth regulator treatment on cocoyam shoot and root proliferation after 4 weeks of subculture in multiplication media.*

Growth Regulator Level (μM)		Shoot No.**	Root No.**
BAP	TDZ		
0	2	17.5 a	1.1 a
20	2	9.8 a	4.0 a

* Shoot tips had been initiated on 5μM BAP and subcultured twice before in the same media prior to transfer.

** Values are the average of 20 replicates. Means followed by the same letter within a column are not significantly different at the 5% level of significance with Tukey's Multiple Range Test.

significantly greater in number than those in cytokinin supplemented media (Table 27). These results, in stationary liquid media, contrast with those obtained in solidified media in an earlier experiment (Table 13). Further investigations are required to substantiate this finding.

Shoots rooted profusely when removed from the influence of TDZ. These results are evident in Table 27, where no roots were formed on TDZ treatments as compared to GRF. The responses of 2μM TDZ alone and in combination with 20μM BAP to root formation were not significantly different (Table 26).

D. BEHAVIOUR OF COCOYAM PLANTLETS IN THE GREENHOUSE FOLLOWING VARIOUS ACCLIMATIZATION TECHNIQUES

Cocoyam plantlets were transferred to the greenhouse during summer and winter months. The environmental conditions as well as treatments were different at these times. In all cases, 100% survival of plantlets was obtained, but the acclimatization treatments differred relative to leaf damage. Except for a few plants in the control treatment that suffered complete wilting of all leaves, no severe desiccation was observed. It was evident that roots and the vascular system were functional as reflected in absorption and transport of water and nutrients with new leaf development.

Table 27. Comparative effect of growth regulator-free and multiplication media on cocoyam shoot and root proliferation at 4 weeks from subculture.*

Medium	Shoot No.**	Root No.**
Multiplication	10.0 b	0.0 b
Growth Regulator-Free	17.2 a	5.2 a

* Shoot tips had been initiated on 5μM BAP and subcultured twice in the same growth regulator multiplication media prior to transfer onto the above media.
** Figures are means of 20 replicates. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

1. Summer Acclimatization

1.1. Leaf Damage of Plantlets on the Open Bench

Cocoyam plantlets with or without acclimatization after transplantation showed no significant desiccation effects one hour after transfer to the open bench. This is illustrated by the percentage of damaged leaves and the wilting assessment (Tables 28 and 29). However, differences were observed 24 hours later and in subsequent periods of observation. Leaf damage was characterized by initial wilting starting from the edge to almost half-way across the lamina. Plantlets acclimatized by uncapping the culture tube showed 14.9% leaf injury after 24 hours of acclimatization, as compared to only 0.6% in plantlets acclimatized under mist (Table 28). Similarly a wilting assessment rate of 3.6 and 4.0 for tube and mist acclimatization, respectively (Table 29) were observed. A majority of the affected leaves in the uncapped tube treatment were those that had turned yellow during acclimatization. Plantlets acclimatized in the humidity tent sustained 6.0% leaf injury after 24 hours (Table 28). Percentages of damaged leaves were not significantly different after 24 hours from acclimatization for treatments of mist, humidity tent and control (Table 29).

At one week from acclimatization, more wilting was observed as the percentage of damaged leaves increased significantly for open tube acclimatization (43.7%) as compared to that for humidity tent (15.9%) (Table 28). Associated with the number of leaves damaged was a wilting assessment rate of 2.8 and 3.5 for plants acclimatized in tubes and humidity

Table 28. Effect of acclimatization procedures on leaf damage of cocoyam plantlets held on the open bench with time.

Treatment	% of Damaged Leaves*				
	24 hrs	1 Week	2 Weeks	3 Weeks	4 Weeks
Tent	6.0b	15.9b	10.4b	5.8c	2.1c
Mist	0.6b	32.2a	20.0b	13.2c	10.0b
Tube	14.9a	43.7a	37.5a	19.1ab	15.8ab
Control	7.5ab	34.2a	37.1a	23.2a	20.8a

* Figures are means of 42 observations. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 29. Relative wilting of cocoyam plantlets upon transfer to the open bench after 5-day acclimatization with time.

Treatment	Wilting Assessment*				
	24 hrs	1 week	2 weeks	3 weeks	4 weeks
Tent	3.8ab**	3.5a	3.6a	3.8a	3.9a
Mist	4.0a	2.9b	3.3ab	3.5ab	3.6b
Tube	3.6b	2.8b	3.0bc	3.4b	3.5bc
Control	3.8ab	2.9b	3.0c	3.2b	3.3c

* Wilting assessment of leaves ranged from 0 (dead) to 4 (intact).
** Figures are means of 42 observations. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

tent, respectively (Table 29). Generally, fully expanded leaves wilted, while the youngest leaves did not. Newly formed ones did not show signs of stress. The peak of damage to leaves occurred one week after acclimatization (Table 28), a time when plants were expected to be established. Wilting in most instances was irreversible, so damaged leaves persisted after this period until they were shed.

By the second week after acclimatization, most plants in all groups had formed new leaves and shed the oldest ones (Table 30). This was evident in a decrease in the percentage of damaged leaves at two weeks, three weeks and four weeks after acclimatization, as compared to previous periods (Table 28). Plants from mist and humidity tent acclimatization treatments suffered significantly less injury than the uncapped tube and control plants. These significant differences emphasize the importance of the method of acclimatization, and its effect on plantlet establishment.

Wilting was not immediate, but occurred gradually, even in the control plants without an acclimatization treatment. Mist treatment delayed wilting, but when the plants were subjected to normal greenhouse conditions they showed even greater wilting after one week than those plants subjected to the high humidity treatment (Table 29).

1.2. Effect of Acclimatization Techniques on Growth Habit

After acclimatization plants continued to grow actively in the greenhouse with apparently normal leaf and plant morphology. One main characteristic of cocoyam is the shedding of older leaves as new ones are

Table 30. Effect of acclimatization procedures on leaf initiation of tissue culture-derived cocoyam plants after 2, 4 and 6 weeks.

Treatment	No. of Leaves Initiated*		
	2 Weeks	4 Weeks	6 Weeks
Tent	1.2 a	2.2 a	3.5 a
Mist	0.6 c	1.6 b	2.8 c
Tube	0.9 b	2.1 a	3.4 ab
Control	0.6 c	1.8 b	3.0 bc

* Leaf number is a cumulative mean of 42 observations. Means followed by the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

formed. Therefore, plant growth was evaluated by the number of new leaves produced rather than measuring plant height. Significant differences were found in the number of new leaves formed in plants among the different acclimatization treatments (Table 30). Most plants had produced at least one new fully expanded leaf by the second week after acclimatization. This indicates that cocoyam plantlets can be easily acclimated to more normal environmental conditions. There was no correlation between the number of roots present at transplantation and leaf formation (data not shown).

Two weeks following acclimatization, an average of 1.2 leaves were produced from plants acclimatized in the humidity tent, as compared to only 0.6 leaves in mist-acclimatized and control plants (Table 30). An average rate of one new leaf per plant was produced every two weeks. Those plants from the mist system produced fewer leaves than the other treatments at four and six weeks; this was significantly different from those acclimatized in humidity tent and uncapped tubes.

The relatively poor growth performance of plants acclimatized by mist may be attributed to the wet conditions they were subjected to. Griffis et al. (1983) reported that nutrients are leached under a misting system, and that the wetness creates an environment favorable for growth of microorganisms. Moreover, cocoyam, unlike taro, cannot withstand waterlogging under natural conditions (Onwueme, 1978; Caveness et al., 1986; FAO, 1988). Continuous misting for a period of five days, in addition to the high humidity, may have been too wet to ensure normal growth. However, the overall trend

was that more leaves were produced than shed (Tables 30 and 31). Reduction in growth upon transplanting of tissue culture plantlets has been frequently reported in the literature (Grout and Aston, 1977b, 1978; Grout and Millam, 1985).

An evaluation of the number of leaves shed per plant per treatment is an indication of the reverse of leaf production. Two weeks following acclimatization, plants subjected to the humidity tent treatment shed only an average of 0.8 leaves as compared to 1.3 for the control plants (Table 31). Significance was observed in the first two weeks, but not in the subsequent weeks. At four and six weeks, leaf turnover was not significant among treatments. This is an indication that once adapted the natural shedding of leaves occurs independent of the method of acclimatization. However, the number of leaves shed was comparatively lower than that encountered from non-tissue culture-derived plants under field conditions (Spence, 1970), possibly due to the use of GRs while in culture. Spence (1970) observed that field grown cocoyam plants were wasteful in the manner in which they produced and maintained their leaves, and suggested the use of GRs to alleviate the shedding. The continuous turnover of large leaves reduced photosynthetic productivity of the plants.

The ability to successfully transfer cocoyam plantlets from culture at a relatively low cost with minimal loss, is important to the micropropagation technique, especially at the commercial scale. In general, many tissue culture regenerated plant species are lost during transfer to natural conditions. These

Table 31. Effect of acclimatization procedures on leaf shedding of tissue culture-derived cocoyam plants at 2, 4 and 6 weeks.

Treatment	No. of Leaves*		
	2 Weeks	4 Weeks	6 Weeks
Tent	0.8 a	1.3 a	2.2 a
Mist	1.2 ab	1.6 a	2.2 a
Tube	1.3 b	1.6 a	2.0 a
Control	1.2 ab	1.7 a	2.3 a

* Leaf number is a cumulative mean of 42 observations. Means followed by the same letter whthin a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

losses are associated with rapid water loss and desiccation during the acclimatization phase. Mist systems and humidity chambers are most commonly utilized, but plant loss may still occur (Wardle et al., 1983). Short et al. (1987) evaluated the success of a micropropagation system by the percentage of plants that are successfully transferred from culture to natural soil conditions.

Virtually all cocoyam plants transferred from culture to in vivo conditions survived, even with no special in vitro acclimatization provided. Onokpise et al. (in press) has also undertaken acclimatization studies with tissue culture-derived cocoyam plantlets directly transferred into various soil substrates and obtained 100% survival in all treatments, acclimatized under high humidity for three days. Staritsky et al. (1986) reported that rootless cocoyam shoots could be easily rooted and would rapidly develop into plantlets when transferred into soil. The present findings are supportive of the fact that the transfer and establishment of cocoyam plantlets from culture to soil do not require special treatments. Acclimatization procedures may be either unnecessary or just advantageous for a short period, especially in areas such as the humid tropics with relatively high humidities. Otherwise, a humidity tent or cheaper method of maintaining a moderately high humidity is recommended, rather than an expensive misting system, in areas with low relative humidities.

2. Winter Acclimatization

2.1 Leaf Damage of Plantlets on the Open Bench

Plant leaves were slightly wilted during transplantation, but plants acclimatized under mist and humidity tent regained turgidity. Plants transferred directly from culture to the open bench were more stressed after 24 hours than their acclimatized counterparts (Table 32). This poor performance of the plants as compared to summer transplantation may be related to the harsh environmental conditions encountered at the time of transplantation. The plants may have been sensitive to the relative humidity of 42% and temperature of 22°C that contrasted with the culture conditions of 90-100% relative humidity and 25 $\pm$ 3°C. Most wilted leaves soon regained turgidity. Plants acclimatized under mist and in the humidity tent had wilted leaves only after one week of acclimatization, which was gradually reduced in subsequent weeks (Table 32). Once again the critical period for leaf injury occurred during the first week after acclimatization.

Holding the plants under mist or high humidity for five days before transfer to the open bench did not affect the survival rate of the plants, but did affect the damage caused to leaves. All plants survived in all treatments, but the results with mist or humidity tent was always superior to that of the control. The presence of BAP or GA3 in the elongation media did not effect any significant difference in wilting at any of the observation times (Table 33). Thus the GRs did not evidently influence transpiration or related processes in cocoyam.

Table 32. Relative wilting of cocoyam plantlets upon transfer to the open bench after acclimatization with time.

Treatment	Wilting Assessment*			
	24 Hours	1 Week	2 Weeks	4 Weeks
Tent	4.0 a**	3.7 a	3.9 a	4.0 a
Mist	4.0 a	4.0 a	4.0 a	4.0 a
Control	1.4 b	2.9 b	3.4 b	3.8 b

* Wilting assessment of damaged leaves ranged from 0 (dead) to 4 (intact).
** Means of 46 observations, and those followed by the same letter are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 33. Relative wilting after acclimatization of cocoyam plantlets elongated on different growth regulators upon transfer to the open bench with time.

Growth Regulator	Wilting Assessment*			
	24 Hours	1 Week	2 Weeks	4 Weeks
BAP	3.1 a**	3.5 a	3.7 a	3.9 a
GA3	3.1 a	3.6 a	3.8 a	3.9 a

* Wilting assessment of damaged leaves ranged from 0 (dead) to 4 (intact).
** Means of 69 observations averaged over three acclimatization treatments. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

2.2 Effect of Acclimatization Techniques on Growth Habit

The growth and development of plants was not affected by the method of acclimatization, nor by the GR used during shoot elongation. New leaves were produced by the second week after acclimatization (Tables 34 and 35), and more grew by four weeks. The rate of formation, however, was low as compared to summer plants (Table 31) except for the mist treatment. This lag in growth could be attributed to the low temperatures, humidity, and lower light intensities in winter conditions within the greenhouse which probably slowed conversion from heterotrophic to autotrophic nutrition. These unfavorable conditions slowed development but did not lead to the death of any plants.

3. REGENERATED PLANTLET CHARACTERISTICS

Tissue culture regenerated cocoyam plants did not show any obvious deviations, and were morphologically similar to their conventionally propagated counterparts (Figure 11). The plants retained the characteristic sagitate leaves of conventionally-propagated plants without modifications of color or leaf shape. On rare occassions, twin plants which shared a common petiole but different laminae were observed in vitro in TDZ media. However, when separated into individual plants at the pretransplantation stage, they developed normally. It has been reported that plants regenerated from axillary shoots are genetically stable, unlike those from adventitious tissues (Tisserat, 1985; Hussey, 1986). However, Acheampong and Henshaw (1984) did not

Table 34. Effect of acclimatization procedures on leaf formation of cocoyam plantlets with time.

Treatment	No. of Leaves* 2 Weeks	4 Weeks
Tent	0.4 a	1.3 a
Mist	0.8 a	1.6 a
Control	0.6 a	1.5 a

* Leaf number is a cumulative mean of 46 observations. Figures followed by the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 35. Effect of growth regulators in vitro on subsequent leaf formation of cocoyam plantlets after acclimatization.

Growth Regulator	No. of Leaves* 2 Weeks	4 Weeks
BAP	0.6 a	1.5 a
GA3	0.6 a	1.4 a

* Leaf number is a cumulative mean of 69 observations averaged over the three acclimatization procedures. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Figure 11. Conventionally-propagated and tissue cultured (3 months in greenhouse) cocoyam plants growing in the greenhouse.

find chromosomal aberrations in the somatic tissues of root tips of cocoyam plants regenerated from adventitious tissues. A similar assumption could be made for plants derived from axillary shoots. However, in order to confirm the reproducibility of a cultivar, genotypic and histological analyses should be conducted. The plants regenerated in vitro from this study did tuberize by the fourth month after transplantation (data not shown).

Dasheen Mosaic Virus (DMV) disease, common in propagules, was not visually detected in vitro and in young acclimatized plants. Some characteristic symptoms, which included chlorosis and distorted leaves, were readily detected in a few plants (<1%) approximately four months after transplantation. This would indicate that the disease was not completely eliminated, and the plants cannot be described as disease-free without being screened, or that they were reinfected from traditionally propagated plants maintained in the greenhouse. Procedures to eliminate viruses from cocoyam tissues in vitro have been established using submillimeter shoot tips (Hartman, 1974; Licha et al., 1980; Lui et al., 1982, 1988; Nguyen and Nguyen, 1987). It is hoped that the procedures herein developed could serve as an invaluable tool to speed up the propagation of elite materials with desirable qualities, but screening of tissue culture derived plants by serology or electron microscopy techniques is required before release. Verified clean stock should increase yields and facilitate transportation across national borders to areas where the need is greater.

E. COMPARATIVE STUDY OF THE NUMBER OF STOMATA IN TISSUE CULTURE-DERIVED AND CONVENTIONALLY-PROPAGATED PLANTS

1. Stomatal Frequency

Cocoyam leaves from all sources were amphistomatic (Table 36). However, almost twice as many stomates were found on the abaxial surface as on the adaxial surface (Table 37). Similar observations were reported for *Rubus idaeus* (Donnelly and Vidaver, 1984). Stomatal frequency (SF), the number of stomates per mm^2, was significantly greater in the non-micropropagated control plants than in the acclimatized greenhouse plants (Table 36 and Figure 12). In vitro plants had an average SF that was not statistically different from those of acclimatized and conventionally-propagated plants.

The reduced SF in leaves of acclimatized plants may have been due to their enlargement. Blanke and Belcher (1989) noticed a drastic decrease in SF of transferred apple plants, which was attributed to leaf expansion. In strawberry plants, increase in size of persistent leaves was mainly the result of cell enlargement, rather than increase in cell number (Fabbri et al. 1986). On the other hand, Brainerd et al. (1981) reported significantly reduced cell length in the upper epidermis of transferred 'Pixy' plum plants as compared to those aseptically and field grown. Comparable findings, where SF was greater with in vitro plantlets than those that had been removed from culture were observed in *Liquidambar styraciflua* (Wardle et al., 1983; Wetzstein and Sommer, 1983), and apple (Blanke and Belcher, 1989).

Table 36. Stomatal frequencies of leaves of in vitro, acclimatized and conventionally-propagated cocoyam.

Plant Source	Stomatal Frequency*	
	Abaxial Surface	Adaxial Surface
In Vitro	33.0 ab	17.9 ab
Acclimatized	20.5 b	11.9 b
Conventional	38.9 a	22.4 a

* Figures are means of 10 observations, and those followed by the same letter in a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Table 37. Stomatal frequencies and indices of leaf surfaces of cocoyam plants.

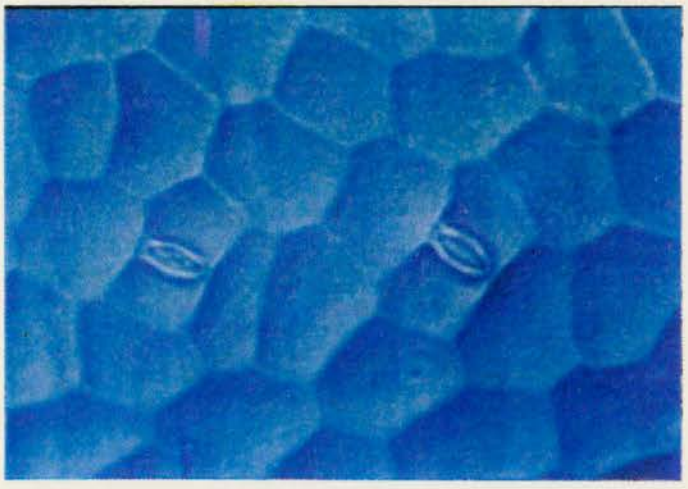
Leaf Surface	Stomatal Frequency*	Stomatal Index*
Abaxial	30.5 a	8.2 a
Adaxial	17.4 b	5.0 b

* Values are means of 30 observations. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

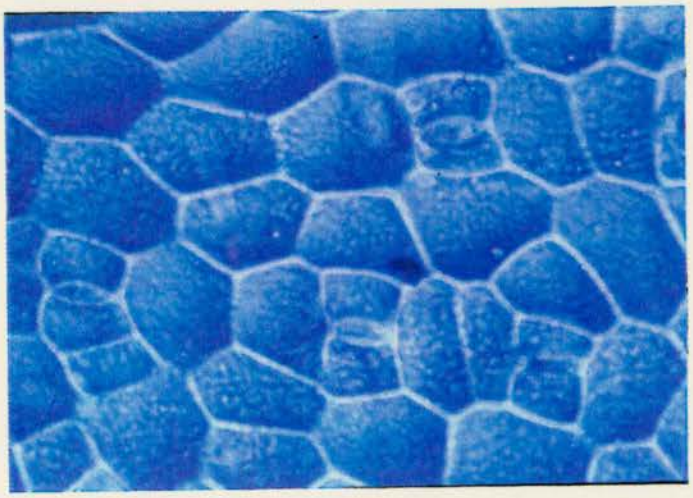
Figure 12. Photomicrographs of leaf imprints from abaxial surfaces of cocoyam plants.
A. In vitro plantlets - X600.
B. Acclimatized plants - X600.
C. Conventionally-propagated plants - X600.

A

B



C



The average SF of 17.4 and 30.5 for adaxial and abaxial surfaces of cocoyam, respectively, are relatively low, when compared to 27.5 and 150 for *Rubus idaeus* (Donnelly and Vidaver, 1984), 184.5 (adaxial only) for *Vitis* sp. 'Valiant' (Dami, 1991), and 225 $\pm$ 60 (adaxial only) for *Prunus insititia* (Brainerd et al., 1981). This low SF may have contributed to low transpiration rates, which resulted in less wilting and high survival rates of cocoyam plantlets after transplantation. In contrast, Brainerd and Fuchigami (1981) suggested that the high SF of apple micropropagated plantlets was responsible for the higher water loss observed. Brainerd et al. (1981) also showed that a marked difference existed in the SF of cultured 'Pixy' plum (150 $\pm$ 60 stomata per mm^2) as compared to the transferred plantlets grown in the greenhouse (300 $\pm$ 60 stomata per mm^2). Even with this low SF, they observed a more rapid water loss from the in vitro plantlets than their greenhouse counterparts, which was attributed to water stress injury. However, the rapid water loss may have been due to stomatal malfunction (Brainerd and Fuchigami, 1981), or the size of the stomates (Wetzstein and Sommer, 1983). Wetzstein and Sommer (1983) found that stomata were also larger in in vitro plantlets of sweetgum, in addition to their greater densities. As indicated by Brainerd and Fuchigami (1981) and Conner and Conner (1984), stomates have a greater part in water loss of plantlets than epicuticular wax.

Epidermal cells (EC) of cocoyam leaves from the three sources were polygonal or irregular with undulate anticlinal walls (Figure 12). However, the

anticlinal walls were less distinct in ECs of cultured plantlets. The cells were also varied in size and shape. Stomates of greenhouse plants were elliptical and sunken, while those of in vitro plants were more spherical and raised, but below the level of epidermal cells (Figure 12). Stomates from all leaf sources were scattered and at unequal distances from one another. However, ECs and stomata along the veins were smaller and aligned in a stream-like manner. Abaxial and adaxial stomata were similar to one another, with varying sizes on both sides.

2. Stomatal Index

Since there was a great variation in stomatal size among the different plant types, SF was not an appropriate estimate for comparison (Conner and Conner, 1984). Stomatal index (SI) is the number of stomata / (number of epidermal cells and stomata) x 100 per mm^2. No significant differences among SI were found in the cocoyam leaves obtained from the various sources (Table 38). These results corroborate previous findings which were reported for *Solanum laciniatum* (Conner and Conner, 1984), *Rosa multiflora* (Capellades et al., 1990), and *Vitis* sp. 'Valiant' (Dami, 1991) in comparisons made between in vitro and field grown plants. Dami (1991) found significantly greater stomatal densities in greenhouse-grown plants than in in vitro cultured leaves, but found none when SI comparisons were made. These results agree with the idea that SI is a better estimate than SF in comparisons involving leaves with stomates of different sizes (Conner and Conner, 1984;

Table 38. Stomatal indices of cocoyam leaves from in vitro, acclimatized and conventionally-propagated plants.

Plant Source	Stomatal Index*	
	Abaxial Surface	Adaxial Surface
In Vitro	8.0 a	4.6 a
Acclimatized	8.2 a	5.0 a
Control	8.6 a	5.3 a

* Values are means of 10 observations. Means followed by the same letter within a column are not significantly different at the 5% level with Tukey's Multiple Range Test.

Donnelly and Vidaver, 1984; Dami, 1991). However, SF may more reliably estimate transpirational water loss.

F. COMPARATIVE STUDY OF EPICUTICULAR WAX CONTENT ON LEAVES OF TISSUE CULTURE-DERIVED AND CONVENTIONALLY-PROPAGATED PLANTS

Cocoyam plantlets cultured in vitro were found to have greater deposits of epicuticular wax (EW) (Table 39). Gravimetric determination showed in vitro leaves to have an average of 88.6 $\mu g/cm^2$, as compared to 50.1 $\mu g/cm^2$ for plantlets transferred and grown in the greenhouse (Table 39). This amount was significantly different from acclimatized plants, but was similar to the non-micropropagated control plants. Similar results were obtained in a repeat of the experiment. Apparently, there was less in wax deposits per unit area after transplantation. Sutter (1988) observed a similar phenomenon with apple plants, with more EW in vitro and less after acclimatization. She suggested that the decrease may have been related to two possible causes: leaf enlargement that exceeded the synthesis of additional wax to cover the additional surface area; and wax metabolism during acclimatization, since previous studies have shown that wax biosynthesis and degradation is a continual and dynamic process (Cassagne and Lessire, 1974). These results are in contrast to reports where more extensive wax deposits were observed in greenhouse and field plants than observed in vitro. Examples include cauliflower (Grout, 1975), carnation (Sutter and Langhans, 1979), cabbage

Table 39. Effect of plant source on epicuticular wax (EW) formation of cocoyam leaves.

Plant Source	Epicuticular Wax ($\mu g/cm^2$)*
In Vitro	88.6 a
Acclimatized	50.1 b
Control	74.5 ab

* EW is the average of two experiments. Means followed by the same letter in a column are not significantly different at the 5% level, using Tukey's Multiple Range Test.

(Sutter and Langhans, 1982), strawberry (Fabbri et al., 1986), chrysanthemum (Sutter, 1988) and grape (Dami, 1991).

Several factors may affect wax deposition. Sutter and Langhans (1979; 1982) and Wetzstein and Sommer (1983) attributed the reduced amounts of wax formed in vitro to high humidity and the low light conditions of the cultures. This reasoning was substantiated when wax deposition was increased in vitro by reducing the relative humidity in the culture vessel (Grout and Aston, 1977a; Wardle et al., 1983; Ziv et al., 1983; Short et al., 1987; Marin et al., 1988; Smith et al., 1990; Capellades et al., 1990). Humidity, radiant energy and temperature were also shown to influence wax density and dimension (Whitecross and Armstrong, 1972). Significantly higher wax content occurred in plants grown under high light intensity such as sunlight, when compared to their counterparts under shade (Whitecross and Armstrong, 1972; Salisbury and Ross, 1985).

Wax deposition after plantlet transplantation occurs with time. Wax formed after 10-14 days in *Brassica oleracea* (Grout and Aston, 1977a; Wardle et al., 1979) and 17/18 days in carnation (Sutter and Langhans, 1979). Fabbri et al. (1986) observed an increase in EW deposits of transferred strawberry plantlets during the first 20 days, while similar findings were observed in *Solanum laciniatum* acclimatized plants after a month (Conner and Conner, 1984). The results obtained in this study showed a decrease in wax content per unit area in transferred plantlets at 9 and 12

weeks from transplantation. These results which conflict with previous work may indicate that wax deposition and breakdown are species-dependent.

The effect of EW on transpiration rates of in vitro and transferred plantlets have contradictory reports with various species. Excessive water losses reported for in vitro plantlets have been related to their lack of EW (Grout and Aston, 1977a). An inverse relationship was found between the amount of EW on leaves and rate of water loss for cauliflower (Grout and Aston, 1977a; Wardle et al., 1983), and chrysanthemum (Smith et al., 1990). This correlation was substantiated in cabbage, when greenhouse-grown leaves lost significantly more water after removal of EW by chloroform (Sutter and Langhans, 1982). However, the relationship has not been found to be true for all species (Sutter 1985, 1988).

Sutter and Langhans (1979) and Wetzstein and Sommer (1982) indicated that the environment in which a plant grows determines its morphology and chemical composition. The in vitro conditions in which cocoyam plantlets were grown seemed favorable for EW formation. This high EW content may have contributed to plantlet survival upon transfer ex vitro. However, Brainerd and Fuchigami (1981) and Conner and Conner (1984) showed that EW was less important than stomates in determining the amount of water loss in plants. The leaf cuticle was only effective in controlling water loss after stomatal closure (Conner and Conner, 1984). The sunken and ellipsoidal stomata of cocoyam leaves in vitro, in addition to their high EW content, may have been invaluable in conferring plantlet survival. Further

studies to evaluate stomatal functioning are necessary to determine whether stomates of plantlets close when stressed.

The low wax content of acclimatized plants may have been caused principally by the rapid leaf expansion that surpassed wax formation. The environmental conditions were not optimum (Sutter and Langhans, 1979; Wetzstein and Sommer, 1982), but did favor wax formation in vitro. Another possible cause for the high amounts of EW observed in vitro may have been the dissolution of internal lipids from open stomates. Several researchers have reported the characteristic inability of stomata of in vitro plants to close upon removal from culture (Brainerd and Fuchigami, 1981; Wetzstein and Sommer, 1983; Conner and Conner, 1984). This could be true for cocoyam. It could also relate to the fact that cocoyam typically grows in high humidity, and thus may have wax production even under high humidities.

CHAPTER V

SUMMARY AND CONCLUSION

Cocoyam has been successfully regenerated in vitro (Hartman, 1974; Acheampong and Henshaw, 1984; Lui et al., 1988, Cedeno-Maldonado et al., 1988; Wutoh, 1989; Onokpise et al., in press) but most report regeneration via adventitious buds, Hartman (1974) and Acheampong and Henshaw (1984). Hartman (1974) regenerated cocoyam plantlets via callus while Acheampong and Henshaw (1984) produced an average of 1.5 plantlets per culture from protocorm-like bodies. However, Acheampong and Henshaw (1984) required an initiation period of 18 weeks. The problem with regenerated plantlets from adventitious tissues is that they are often associated with a genetically variable population (Gupta, 1985; Hussey, 1986; Torres, 1989; Debergh and Read, 1991).

Reports of direct regeneration from shoot-tips of cocoyam also showed low propagation rates of 1-1.5 plantlets per culture (Cedeno-Maldonado, 1988; Wutoh, 1989; Onokpise et al., in press). However, Wutoh (1989) and Onokpise et al. (in press) employed larger explants (5-25mm) on a growth regulator-free medium and were able to produce plantlets within six weeks of initiation.

The present study has demonstrated that cocoyam plantlets can be mass produced in vitro through axillary branching of cultured shoot-tips. This was accomplished by overcoming apical dominance using BAP and TDZ.

This study also demonstrated that cocoyam can be micropropagated in vitro through various procedures: the use of either BAP, TDZ or both; agitated and stationary liquid media at the initiation stage; agitated, stationary or semi-solid (after the first multiplication phase) media at the multiplication stage; and stationary liquid and semi-solid media at the elongation and rooting stage. Shoot proliferation in all procedures was low at initiation, but increased substantially during the multiplication phases. There was thus an influence of subculture on shoot proliferation. The number of shoots produced at the initiation phase of six weeks was comparable to those previously reported in the literature. However, much larger numbers were more quickly produced during the multiplication stage.

A comparison of the effect of BAP and TDZ on axillary shoot production showed that all concentrations tested (5-20μM BAP and 1-4μM TDZ) produced significantly more axillary shoots per shoot tip than the control without cytokinins, as well as those reported in the literature. At 10 weeks from initiation on agitated liquid media, an average of 7.37 and 8.58 shoots per explant were obtained from 20μM BAP and 2μM TDZ, respectively (Table 10). Shoots from the BAP treatment produced extensive branched roots with profuse root hairs. Hence without further culture, an average of 8.58 plantlets could be safely transplanted into soil after only 10 weeks from

initiation. This rate is higher and more rapid than any procedure previously reported in the literature. However, greater proliferation rates, 9.68 and 13.05, were obtained through the use of 20μM BAP and 2μM TDZ, respectively, 12 weeks from initiation (Table 10). Rates of shoot proliferation continued to increase in 2μM TDZ after 14 and 16 weeks from initiation, but increased only slightly in BAP up to 14 weeks and levelled off thereafter (Figure 6). These cytokinin levels overcame the apical dominance effect, thus stimulating the development of axillary buds of both the main shoot and the subsequent axillary shoots formed.

Subculture into fresh multiplication media gave an average of 30 explants per culture after six weeks. Continued subculture of each explant would give substantial number of shoots. Shoots produced with BAP were larger and more normal in appearance than those produced with TDZ, which were small, compressed and stunted. Shoot clusters continued to proliferate irrespective of the presence or absence of growth regulators. However, when isolated into single shoots (<5mm) in growth regulator-free medium, individual plantlets with well defined shoot and root systems developed within two weeks. The ease of proliferation, elongation and rooting shoots in a growth regulator-free medium, illustrates the theoretical possibility of regenerating more than one million plants from an explant in a year. The success of propagule multiplication even with different procedures reiterates the consistency of micropropagating cocoyam through axillary shoot enhancement, although the formation of a few protocorm-like bodies was observed.

Based on the results of the experiments described herein, the use of stationary liquid media is recommended because of economic reasons (Table 1). Any of the cytokinin treatments described in the materials and methods would be adequate, however, the effects of 2μM TDZ alone or in combination with 20μM BAP at the initiation stage on subsequent proliferation were outstanding, especially when transferred into a multiplication medium with 2μM TDZ. Subcultures are recommended at four to six week intervals. At these short intervals, cultures are presumably in a linear growth phase, which may result in a more efficient multiplication scheme that would produce large numbers of plants per year. Individual shoots can be elongated and rooted in a growth regultor-free medium for faster development of whole plantlets. Branched roots and root hairs developed in both agar and liquid media.

Plantlets with a well developed shoot and root system were transferred from culture into non-sterile soil for establishment. A relatively high humidity (60-80%) is necessary for approximately two weeks to prevent leaf injury resulting from wilting and desiccation. All transferred plantlets, irrespective of the size and acclimatization treatment, survived greenhouse conditions during the summer and winter seasons. Plants grew and developed new leaves with characteristics identical to those of conventionally-propagated plants. Based on this uniformity in the greenhouse, it is likely that the plants are true-to-type. However, further studies such as an evaluation of the ploidy level and growth of cormels may be essential to ascertain the characteristics of the plant.

The observation of Dasheen Mosaic Virus (DMV) symptoms on a few plants long after transplantation implies the presence of the virus. Thus, some systematic screening procedures must be used to assess the probability of releasing pathogen-free plants.

Studies of the evaluation of stomatal number showed that cocoyam leaves have few stomates on both abaxial and adaxial surfaces, with fewer on the adaxial surface. The fewer stomates, which are in addition sunken, may have aided in preventing excessive water loss during transplantation. High levels of epicuticular wax found in vitro may have contributed to reduced transpiration rates. It is thus possible that the epicuticular wax on plantlets played an important role in minimizing wilting at the time of transplantation. The reduced amounts on acclimatized plants could be attributed to the rapid cell enlargement in expanding leaves, more rapid than the the rate of wax formation.

The present study was done basically with one cultivar, so an evaluation of other cultivars, especially elite materials from a breeding program should be undertaken.

Due to time constraints, it was impossible to continue subculture into the same multiplication media in order to study the accumulated effect on shoot proliferation. A series of subcultures evaluating the relative number of subcultures possible without the reduction of proliferation potential would be valuable.

Erlenmeyer flasks and test tubes employed in the present study did not prove to be the best culture vessels. It was very difficult to take out the mass of tissue proliferated through the narrow mouth. Wider-mouthed culture vessels such as baby food jars or magenta cubes should be used for further studies. During the multiplication phase on solid media, subculturing could be reduced through the addition of fresh liquid media, which could be used at the elongation phase as well.

Tissue culture derived plants should be grown in the field under normal growth conditions to evaluate trueness-to-type. A comparison of the yields and other growth parameters should be studied between in vitro-derived and conventionally-propagated plants.

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APPENDIX

APPENDIX

ANALYSES OF VARIANCE

Table 40. Effect of media substrate and growth regulator treatments on relative growth of cocoyam shoot-tips at 4 weeks from initiation.

Source	Degrees of Freedom	Mean Square	F	F (0.05)
Substrate	3	3.44	9.39	3.59
Growth Regulator	2	12.32	33.62	3.98
Sub x GR	6	2.24	6.11	3.09
Error	11	0.37		

Table 41. Effect of media substrate and growth regulator (GR) levels on cocoyam shoots initiated at 6 weeks from subculture.

Source	Degrees of Freedom	Mean Square	F	F (0.05)
Substrate	1	220.88	8.62	4.06
GR	2	346.41	13.51	3.21
Sub x GR	2	249.69	9.74	3.21
Error	45	25.64		

Table 42. Effect of initiation growth regulator levels on subsequent cocoyam shoot proliferation while in multiplication media of 2μM TDZ or growth regulator-free media.

Source	Degrees of Freedom	Mean Square	F	F (0.05)
Initiation Levels	5	205.16	1.44	2.30
Mult. Levels	1	12319.94	86.72	3.94
Ini x Mult	5	404.88	2.85	2.30
Error	103	142.07		

Table 43. Effect of growth regulator treatments at the multiplication stage on cocoyam shoot proliferation during the elongation stage at 4 weeks from subculture.

Source	Degrees of Freedom	Means Square	F	F (0.05)
Multiplica-tion Levels	7	3188.04	31.19	2.05
Elongation Levels	1	1486.31	14.54	3.89
Mult. x Elon.	7	2.30	2.30	2.05
Error	211	102.23		