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Performance of tea (*Camellia sinensis* L.) on culture media modified with locally sourced substitutes

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1 SUMMARY

This study aimed to develop a suitable, economical, biologically degradable, non-toxic, from locally available naturally occurring constituents for low-tech biotechnological procedures in resource poor developing nations. Tea (*Camellia sinensis* L) whole immature and mature embryos and their respective component parts (embryo axis and cotyledons) were subjected to invitro manipulation on Murashige and Skoog (MS) medium modified with locally sourced substitutes of plant, sea and soil origin. Natural alternatives explored were earthworm cast (EWC), Trona (T) and sea water (SW). Each of this replaced the MS salts while honey replaced the vitamins in MS. Sugarcane replaced sucrose while cassava starch substituted for agar. Explants were scored for survival (SV), plantlet formation (PF) and for somatic embryogenesis (SE). Honey did not effectively substitute the MS vitamins but the sugarcane was the best and most suitable among all the substitutions made. Mature embryos turned green on the earthworm cast based media without germinating; it turned pink in SW indicating low level of anthocyanin synthesis. General trends showed that SW medium was superior to EWC medium while trona medium was poorer. None of the natural media formulated equated the effectiveness and characteristics of the standard MS salts formulation. A major observation is that there are poorer SE development and very slow growth of the plantlets, SE became dormant at the globular or heart shaped stage of embryogenesis. The ultimate goal of this study was to develop a procedure for compounding a natural nutrient medium for tissue culture. The results indicate that there is potential to use natural compounds for tissue culture media to reduce pollutants that are generated as wastes from various laboratories and industries.

2 INTRODUCTION

Plant tissue culture is the aseptic growth of cells, tissues, organ and whole plants in artificial or definable media. The advantage of plant tissue culture is enormous and strategic in plant biotechnology. It has had greater significance and impact in developing nations especially Nigeria. Very few tropical economic plants have benefited from this technology due to the high cost and level of sophistication of equipment and facilities, lack of qualified personnel for the specialized techniques, and unavailability of authentic media ingredients.

Although in-vitro factors are very critical to successful culture of explants, the most important factor is the culture media. Various formulations have been developed since

1902; the most popular has been that formulated by Murashige and Skoog (1962).

The success of plant tissue culture relies mainly on the controlled culture vessel environment which should ensure high humidity. Nutritional requirements for optimal growth of a tissue in vitro may vary with the plant species and even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige & Skoog, 1962) As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. Some of the earliest plant tissue culture media, for example root culture medium of Gautheret (1939), were developed from nutrient solutions previously used for whole plant culture. White (1943) developed the medium from Uspenski and Uspenskaia's medium (1925) for algea, and Gautheret's medium is based on Knop's (1865) salt solution. All subsequent media formulations are based on either White's or Gautheret's medium. Other than for the Knop's (1865) nutrient solution, which was used by early investigators and is still useful today, few media are initiated based on the result of soil composition analysis. Nevertheless, the foremost commonly used media formulation for herbaceous plants is the Murashige and Skoog (1962) and the techniques have been more or less relatively uniform.

The use of plant biotechnology chemicals with potent mutagenic effects is on the increase. There is therefore a need for a proper awareness of bio safety regulations and efforts should be geared towards identifying and using more naturally occurring media components. The gateway into modern plant biotechnology is through tissue culture. However, there is an apparent slow take-off of plant biotechnology in sub-Saharan Africa. This has been partly due to the high cost involved in setting up standard tissue culture facilities and for the procurement of chemicals needed for medium preparation (Stoltz, 1979; Song, 1982). Early attempts to culture tissues found that plants in cultures were heterotrophic rather than autotrophic. Later, it was discovered that sugar and several complex organic substances such as coconut milk, yeast and fruit juices supported cultures where inorganic chemicals could not. Coconut milk thus became the earliest "ready made natural nutrient medium".

Esan (1992a, 1992b) and Esan (1993) reported on some aspects of tissue culture work then on-going at the Cocoa Research Institute of Nigeria (CRIN) on the search for local natural substitutes for nutrient medium components. Trona was then identified and ranked as a satisfactory replacement for the salts used in MS medium. The primary objective of this investigation was the development of a suitable, biologically degradable, non-toxic, non-elitist plant nutrient medium from locally available, naturally occurring constituents of sea, soil, plant and animal origins. The aim is to reduce costs of materials required to carry out simple low-tech biotechnologica1.procedures through the use of definable substitutes.

3 MATERIALS AND METHODS

Whole mature embryos and their respective component parts (embryo axis and cotyledons) were used as explants. Normal procedures for ensuring aseptic conditions were adopted. Inorganic and organic component alternatives explored were prepared as follows:

3.1 Earthworm cast (EWC) preparation: These casts were collected fresh from a well-established forest at the Cocoa Research Institute of Nigeria (CRIN), and allowed to air-dry on the laboratory bench at room temperature (about 27°C) for a week. These were crushed and pulverised into very fine powdery form using a high speed and dry-blending electric motor. Samples were stored in screw-cap bottles and kept in a desiccator. Two methods were adopted in its use. The first was a direct incorporation of the pulverised earth worm cast into the bulk medium before distribution into containers (M'Carteny or Pedialite bottles). By this method, continuous agitation was necessary during distribution. In the second method, the desired quantity of the earthworm cast was soaked in about 600ml of double distilled water for *60* minutes, after which it was stirred thoroughly and filtered through

a fine nylon cheeze cloth and through Whatman filter paper No. 11 by suction pressure to produce the clear filtrate which was added to other media components.

The most important action of earthworms in agricultural ecosystems is the general conditioning of plant remains for internal microbial decomposition. When earthworms ingest mineral soil particles, predominantly clay minerals, the particles are cemented together by calcium humate formed from ingested decomposing, organic matter of foliage litter and calcite excreted by the calciform glands. These products are excreted as a saturated pasty, poorly aerated faecal wastes called casts (De Vleschauweer & Lal, (1981). The casts are usually richer, than the surrounding soil, in calcium, magnesium and humus potassium silicates. Humus is an organic carbon containing materials on the earth surface occurring in soils. It constitute between 70-80% of the organic matter in most inorganic soils and are formed from the chemical and biological degradation of plant and animal residues and from synthetic activities of micro organisms. The product so formed tend to associate into complex organic structures which are more stable than the starting materials

3.2 Trona (T) preparation: Impure form of the substance was procured from the local market and stored in a dessiccator until ready for use. A supersaturated, hot solution of the Trona was prepared in screw-cap, pedialyte bottles. They were cooled and allowed to stand at room temperature for 12-24 hours. Specific aliquots were used of the supernatant suspension in media preparations.

Trona, the ideal formula being (Na_2CO_3, $NaHCO_3$, $2H_2O$) is a naturally occuring hydrated, complex basic salt; and a sesquicarbonate of sodium containing Na_2CO_3 and NaHCO in equimolar proportions. In its crude form, it contains impurities which include quartz, clays, chlorides and sulphates or admixtures (Walther, 1922; Bateman, 1952; Solaja, 1972; Makanjuola and Bettlestone, 1972; Ankra and Dovlo, 1978). The impurities consist of the following elemental constituents: Si, AI, Fe, Ca, K, S, Cl and Mg. Trona, though erroneously termed potash, contains relatively low quantities of potassium; and it occurs in virtually all saline lake waters all over the world.

3.3 Sea water (SW) preparation: This was collected from a recreational beach (Bar beach) in Lagos, along with some of the beach sand in a transparent glass jar equipped with a cover. It was allowed to stand for a week. Specific aliquots were used from the clear supernatant whenever required after filtering through a Whatman filter Paper No. 11. According to Petrucci (1972) sea water contains enormous quantities of elements, the principal constituents being: CI, Na, SO_4, Mg, Ca, K, HCO_3, Br, H_3BO_3, Sr, I and F in decreasing order of quantities. The ocean water is practically a continuous medium, with fairly predictable physical and chemical properties. More importantly, the relative proportions of salts in the main body of the ocean are fairly constant at all latitudes (Transeau *et al.*, 1940).

In general, sea water has proved to be the best culture medium for most sea water plants and in particular marine algae. It has been used at full strength or in its diluted form (Klein and Klein, 1970). At present, there are many formulations of artificial sea waters which have been developed as a result of difficulties encountered in obtaining unpolluted sea water even in costal area of industrialised nations. The advantages of sea water are that it is an excellent osmotic stabilizer, easy to obtain, cheap to prepare and the osmolarity of each batch can be determined easily. Thus it has been used in the isolation and culture of protoplasts (Schneider, 1984). The Lagos lagoon sea water has about 3.2% salinity and specific gravity of approximately 1.028 at (Mabo, et al., 1988d).

3.4 Cane sugar juice preparation: Fresh sugar cane sticks were procured from local markets. The stems were cleansed by scrapping until the stems were white. The juice was obtained by using a hand-press sugar cane juice extractor. A work bench table mounted wench was also found to be equally useful. Another method was to peel off the hard outer cover of the sugar cane stem and to grate the same to produce a fluffy product which can then be pressed in a cheese cloth, when wrapped, and filtered through Whatman filter paper No. 11. Storage of the juice was avoided.

3.5 Honey: This was used in trials as a substitute for MS vitamin complex. Honey is the nectar and saccharine exudation of plants, gathered, modified and stored as honey in the comb by honeybees. The ingredients reported to be in honey are sucrose, glucose, fructose, maltose, iron, nitrogen, sodium, ascorbic acid (vitamin C), thiamin (vitamin B 1, potassium, folic acid.

To each of the specified quantities of the inorganic component substitutes (alternatives) namely trona, sea water and earthworm cast, was

added NaEDTA, yeast extract, casein hydrolysate, coconut milk (or water) vitamins (as in MS) NAA, BA. The p^H adjusted to 5.7 $\pm$ 0.1 before the addition of agar and autoclaving at 121°C for 20 minutes at 15 PSI. Cultures were stored at 26 $\pm$ 2°C with 16 hour light (3500 Lux). Each treatment was represented by 20 cultures each time and the test was repeated five times over a period of two years. The final medium composition is as presented in table 1.

All explants were scored for survival (SV), plantlet formation (PF) and for somatic embryogenesis (SE) on each of the substitute-based medium namely Sea Water (SW), earthworm cast (EWC) and Trona (T).

Table 1: Composition of natural salt complexes- based nutrient media used for initiating cultures of tea.

Component	Amount per liter
Trona	10-15 ml of prepared solution
Earthworm cast	10-15 ml of prepared solution
Sea water	2.0-4.0 ml of prepared solution
$FeSO_4.7H_2O$	
Na2 EDTA	Exact in MS
NAA	2.0 mg
BAP	0.1 mg
Coconut milk	200.0 ml
Casein Hydrolysate	500.0-1,000.0 mg
Inositol	100.00 mg
MS Vitaminn Complex of Honey	As in MS or 2-10 ml of Pure Honey
Agar or Cassava Starch (1.1) w/w	8,000 mg
Sucrose or Sugarcane juice	30,000.0 mg (or 20-30) ml of sugarcane juice
pH	5.7 + 0.1

4 RESULTS AND DISCUSSION

Survival of embryos occurred as a result of pigmentation (greening) for chlorophyll synthesis. Mature embryos turned green on the EWC - based media without germinating, while they turned pink in the SW - based medium indicating low level of anthocyanin synthesis. Anthocyanin synthesis in cultures of cocoa immature embryos has been reported in relation to high concentration of sugar (or sucrose) in the medium (Esan, 1977; Pence et al. 1981a, b; Kononowicz & Janick, 1984; Pence, 1989).

Sucrose plays dual role in the medium used in the culture of immature embryos. It is a source of energy required for the heterotrophic nutrition of the explants as well as a stabilizing osmoticum. The degree and intensity of the anthocyanin pigmentation has been directly correlated with the concentration in the medium of the source of energy (carbon source). Consequently, the pink colour implies a relatively low level of sugar. This anthocyanin pigmentation may be enhanced by some components of the seawater such as chlorides.

Sugarcane juice substituted for analar sucrose was the most encouraging and best among all substitutions evaluated. So far, honey did not effectively substitute the vitamin (organic) fraction of the MS medium. Tables 2 and 3 summarise the relative survival ability, plantlet and somatic embryo formations of the explants on the media being evaluated.

These values were expressed as percentages of the records made on the full MS medium. Although all the three media were able to sustain survival and regeneration, particularly somatic embryogenesis of tea explants, they were still inferior to the Murashige - Skoog medium, since there was no condition where there was 100% score for survival, plantlet formation or even somatic embryogenesis.

Table 2: Response of tea seed and seed component explants on sugarcane juice substituted for analar sucrose in MS medium.

Plant part	Full MS medium			MS substituted with sugarcane		
Tea explants	SV	PF	SE	SV	PF	SE
Whole mature embryo	40	20	10	30	10	10
Mature embryo axis	80	50	20	70	75	40
Mature embryo cotyledon	75	Nil	30	80	Nil	30

Table 3: Relative growth responses of tea seed/seed component explants on medium formulated using locally sourced natural materials.

	Seawater			Earthworm cast			Trona		
Tea explants	**SV**	**PF**	**SE**	**SV**	**PF**	**SE**	**SV**	**PF**	**SE**
Whole mature embryo	30	10	8	25	10	8	25	10	5
Mature Embryo axis	60	30	20	36	20	20	30	20	10
Mature Embryo Cotyledons	70	nil	20	70	nil	10	45	nil	5

It was observed that none of the modified media formulated so far has equalled the effectiveness characteristics of the MS salt formulation. The general trend observed shows that the SW-based medium was either superior to or equal in effect to the EWC-based medium. The T - based medium was comparatively poorer. A major significant observation in general was that there were poorer developments of somatic embryos as well as very slow growth of the plantlets when cultured on locally source substitutes. Most somatic embryos became dormant at the globular and at the heart shaped stage of embryogenesis. Reculturing therefore became a necessity and a more frequent exercise once structures were formed. However, since somatic embryos took a longer period to initiate, it appears as if the chronological age of the structures from their initiation and development on the primary explants was an important factor. The number of somatic embryos produced was not significantly different from those produced on the full MS medium, which was used as standard.

CONCLUSION

Although the ultimate goal of this study was to evolve a procedure for preparing plant nutrient medium using natural and locally available ingredients, this has not been properly perfected or attained yet. However, it is an indication that there is potential to use natural compounds in media formulation towards reducing costs and pollutants that are generated from various plant laboratories and associated industries.

Robertson (1992) concluded that there is a need for the development of indigenous biotechnologies in developing countries particularly in sub-Saharan Africa. Qualitatively, the inorganic nutrients required for various plant tissues appear to be fairly constant (Bhojwani and Razdan, 1983; Kyte, 1983). Similarly, numerous complex nutritive mixtures of undefined composition, like casein hydrolysate, coconut milk, malt extract, tomato juice, and yeast extract, have also been used to promote the growth of certain calli and organs, as well as in the induction of somatic embryos (Pence *et al.,* 1980; Kononowicz, 1984; Novak et al, 1986; Adu-Ampomah *et al.*, 1988). However, Bhojwani and Razdan (1983) advised against the use of these natural extracts and complexes, since different samples of these substances, especially the fruit extracts, may affect the results because the quality and quantity of the growth-promoting constituents in these extracts often vary with the age of the tissue and the variety of the producing organism. Similarly, it has been suggest that in nutritional studies, the use of agar should be avoided because almost all commercially available agar samples contain impurities, especially of Ca, Mg, and trace elements.

Coconut milk was first used successfully by Van Overbeek *et al.* (1941). Coconut therefore became the first ready made "natural" nutrient medium especially for stimulating embryogenesis (Kyte, 1983).

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