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Full Length Research Paper

Effect of 2,4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana

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Modern biotechnological applications in breeding programmes for economically-important crops like cassava begins with successful establishment of stable and active callus cultures for somatic embryos which can be genetically manipulated and induced into planting materials. We have investigated the effects of explants (leaves, stem petiole and axillary buds), auxin (2, 4-D) concentrations (8, 12 and 15 mg/l) and cultivar in the induction of callus cultures from local cassava varieties in Ghana - *Tuaka, Afisiafi, Afebankye* and *Doku*. Callus formation was successfully induced in all explants (leaf, petiole and auxiliary buds) from the four cassava cultivars. Generally, there were no significant differences in callogenesis of the different explants at the three different 2,4-D concentrations ($p < 0.05$) producing 75% callus for explants. The stem petiole or stem bark produced the least percentage of callus per cultivar at all the auxin concentrations. However, the leaf explants were superior in callus formation in all the cassava varieties. Although, statistically insignificant, the 8 mg/l 2,4-D concentration was visually the best in callus formation from explants both within and across the cassava cultivars. This study had shown that different cassava explants respond differently to tissue culture conditions established for callus culture formation.

Key words: Auxin, callogenesis, callus induction media, cassava *Manihot esculenta* Crantz, 2,4-dichlorophenoxyacetic acid (2,4-D), somatic embryogenesis.

INTRODUCTION

Among all the tuber crops grown in Ghana and other parts of Africa, cassava (*Manihot esculenta*) is the most largely grown and therefore, serves as the major tuber food crop for human consumption and also as animal feed (Kay, 1973). In sub-Saharan Africa, cassava is considered as 'life-blood' because the starchy tuber is consumed as a staple and also supports the starch, textile, paint and pharmaceutical industries (Srinivas, 2007). Thus, anything that affects the production of the cassava crop may have serious consequences in food availability and the economy of the people in such growing areas. It is therefore of no surprise that every year millions of dollars are allocated by governments in these growing areas to combat pests and diseases which affect the yields of the crop. Some challenges of cultivation include low nutritive value of cassava (1.2 to 1.8% crude protein, 0.1 to 0.8% crude lipid, 1.5 to 3.5% crude fibre and 1.3 to 2.8% ash) (Albert et al., 2005) and occasionally accumulations of toxic cyanogenic glucosides (White et al., 1998). This therefore, necessitates the adoption of breeding programmes to introduce new varieties of higher nutritional quality. In Ghana, and to date, traditional breeding methods which involve sexual hybridizations between local and exotic varieties of desired traits remain the only option in generating new varieties. Unfortunately, such methods are ineffective in eliminating cyanogenic compounds, diseases and pests and also, they are time-consuming (Smith and Drew, 1990). As alternatives, modern methods of the cell and tissue cultures allow the overcoming of such barriers. These methods allow production of genetically improved

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varieties by transfer of traits of interest (Schopke et al., 1996; Ye et al., 2000). The use of cassava cell and tissue culture techniques including microcuttings, somatic embryogenesis, androgenesis (anthers culture), gynogenesis (ovary culture), among others has great potential in generating disease-free planting materials for cultivation (Smith and Drew, 1990). A stable and active cell or tissue culture is an essential step for any successful plant breeding programme (Guo and Zhang, 2005). Because different plant species and different explants of some species respond differently to culture treatments (Pareek and Kothar, 2003), specific sets of conditions are necessary for each explants per plant. Although, many laboratories have generated cassava plantlets from somatic embryogenesis using different varieties of cassava (Stamp and Henshaw, 1982; Smith and Drew, 1990), as far as we know, there has been no such work on cassava varieties in Ghana. Even in the case of those that have been exploited elsewhere, not much information is available on the variations different cassava explants undergoing callogenesis. Callus is an important material for establishing embryogenic culture and successful production of it from Ghanaian cassava would offer an opportunity for cultivar generation and germplasm storage. This study aimed to test the effect of factors 2,4-D concentration, explants type and cultivar on the callogenesis expression of cassava.

MATERIALS AND METHODS

Study site, explants preparation and culture conditions

Four selected cassava cultivars commonly grown in Ghana, namely *Doku, Afisiafi, Afebankye* and *Tuaka*, were obtained from the farms of the Crop Research Institute (CRI), Fumesua in the Ashanti Region. Fresh stem cuttings of 20 to 30 cm with 5 to 8 nodes were prepared from mature plants. The cuttings were planted in the loamy soil of the Kwame Nkrumah University of Science and Technology (KNUST) experimental fields in November 2008 (average day temperature of 30°C; ambient average night temperature of 24°C, average day light of 12 h and relative humidity of 55%) and repeated in January 2009 (average day temperature of 27°C; average night temperature of 22°C, average day light of 12 h and relative humidity of 55%). The plants were watered daily with sprinkler. Six-week old shoots were severed from the plants and transported in a vase of water to the laboratory. The plants were surface-sterilized with 20% household bleach (*La Croix*) containing 3.5% sodium hypochlorite and 0.015% Triton-X100 for 20 min (leaf explants) or 30 min (stem petiole explants) and rinsed at least three times with sterile water. All stages of sterilization were done in the laminar flow hood. Sterile leaves and stem petioles were cut into discs and pieces of about 10 and 5 mm, respectively. 5 mm of apical and auxiliary were also prepared. The control cultures consisted of all explalnts cultured in media without phytohormonal supplementation.

Callus culture

The cassava explants were cultured on Murashige and Skoog (MS) medium modified by addition of 2% sucrose, 0.5 mg/l $CuSO_4$ and 0.6% agar (gelling agent) as indicated by Atehnkeng et al. (2005). The pH of the medium was adjusted to 5.7 and media containing one of the auxin concentrations (8, 12 and15 mg/l) were prepared then autoclaved at 121°C for 15 min. The media were cast in sterile glass culture plates and solidified in sterile laminar flow hood. The prepared explants (leaf discs, stem petiole and buds) were aseptically transferred to the MS media plates and sealed with parafilm. The cultures were done in triplicates and incubated in the dark at 25°C and monitored daily over 21-day period to observe callus formation.

Measurement of callusing explants and initiation of somatic embryos

At the end of each culture cycle of 21 days, the callogenic explants number and initiated somatic embryo number was scored. Prior, the length of shoots grown in university KNUST soil was measured in *ex-vitro* conditions.

Experimental design

A 3 × 3 × 4 factorial combination in a completely randomized design with three replications was built. In all, 108 treatments were obtained at the end of each culture. A treatment was constituted of the combination of a 2,4-D concentration, explant type and cultivar.

Statistical analysis

The collected dataset were processed by Prism v.4 (GraphPad Software, Inc.). The effect of three factors, 2,4-D concentration, explant type and cultivar, was tested through the ANOVA. The averages were separated according to Newman-Keuls, Dunnett, Duncan or Bonferoni test at 5% significance level. Prior, the normality of measured distributions and variances equality of analyzed samples were verified.

RESULTS

Effect of cultivar on the expression growth in length of shoots in *ex-vitro* conditions

Two statistically different groups of cultivars were identified. First, composed of *Afisiafi* (0.714 cm/day) and *Tuaka* (0.748 cm/day) cultivars, was characterized by high growth in length of shoots (Figure 1). Second, comprising *Doku* (0.547 cm/day) and *Afebankye* (0.645 cm/day), cultivar differed from three previous cultivars by low growth in length of shoots. The recorded averages ranged from 0.547 cm/day (*Duku*) to 0.748 cm/day (*Tuaka*).

Effect of 2,4-D on the callogenesis expression

Calli were successfully produced from explants from each of the four cassava cultivars commonly grown in Ghana (Figure 2). On the average, the formation of callus from explants of the cassava varieties used occurred 7 days

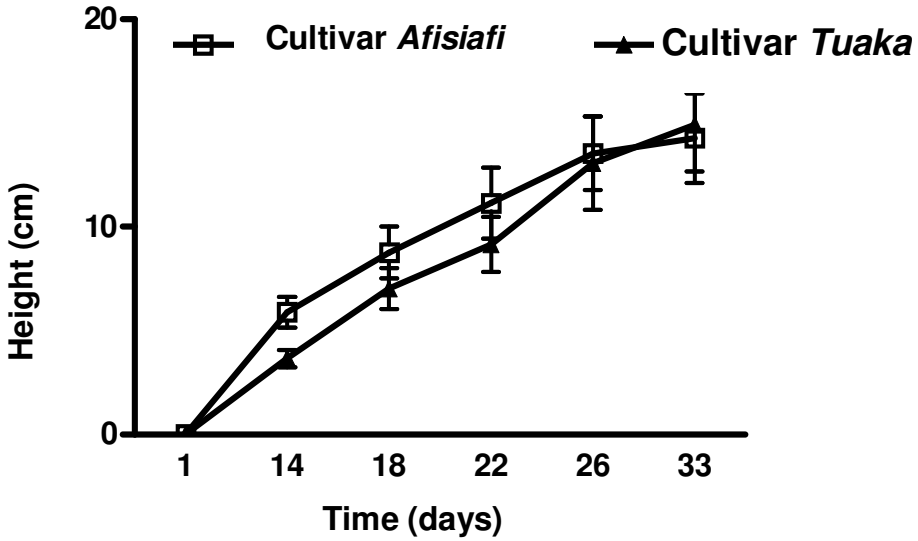

Figure 1. Growth rates (stem increase in cm/day) of cassava cultivars grown in the first 35 days after sprouting. The graph shows growth rates for cassava cultivars *Afisiafi* (filled circles) and *Tuaka* (filled squares). Each represents an average stem height. Vertical traits represent the bars of standard errors of mean.

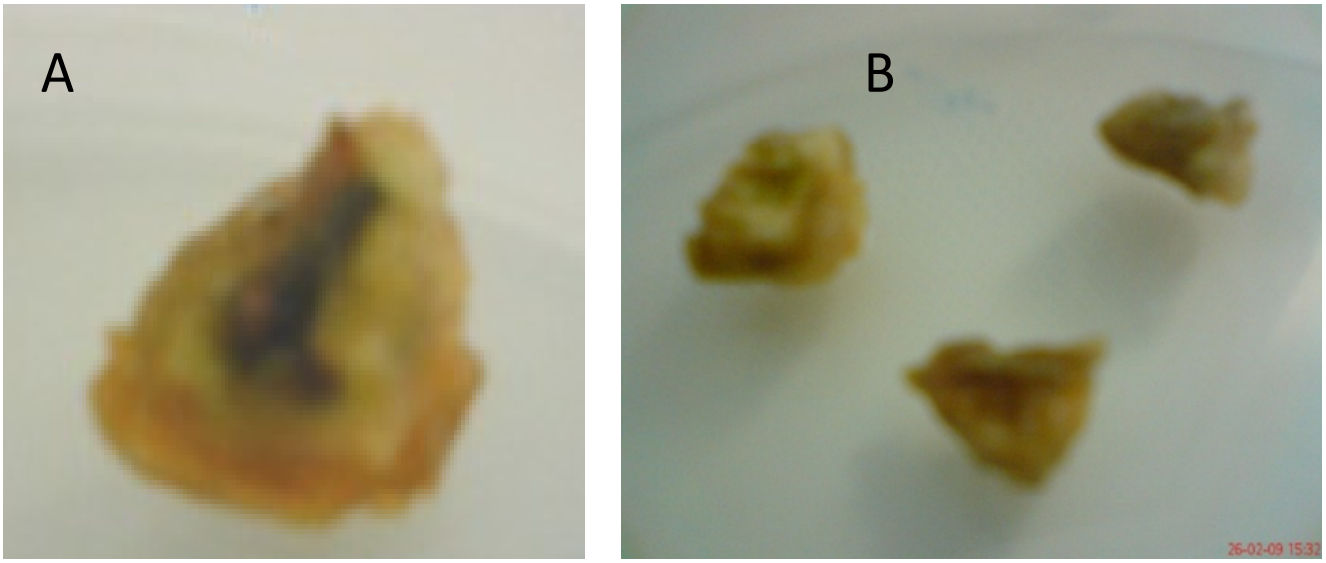

Figure 2. Callogenic explants deriving from stem (A) and bud (B) formed onto MS medium supplemented with 8 mg/l 2,4-D. The bud and stem callus were formed 14 and 21 days, respectively after phytohormone treatment. Bar = 0.5 mm.

after transfer to culture medium. The callusing explants appeared swollen, yellowish-green to brown in color and showed initial growth around the excised portions before enlarging into callus mass. Callusing of leaf explants began with curling of the tissue followed by swelling at the cut edges, while in the case of petioles and buds, the callus was initiated at their terminals. Bark explants from cultivar *Afisiafi* cultured in the medium with 12 mg/l 2,4-D concentration gave the highest percentage of per explants (Figure 2). The stem petiole derived-derived callus, unlike the leaf and bud-callus, were hard callus.

Different percentages of callus formation, ranging from 0 to 75% were observed for different explants (leaves, bark and buds) at the three different auxin concentration used (8, 12 and 15 bmg/l 2,4-D). The cultivar *Tuaka* did not show any significant difference between the percentage callus produced from the explants (leaf, stem petiole and buds) at all the three 2,4-D concentrations

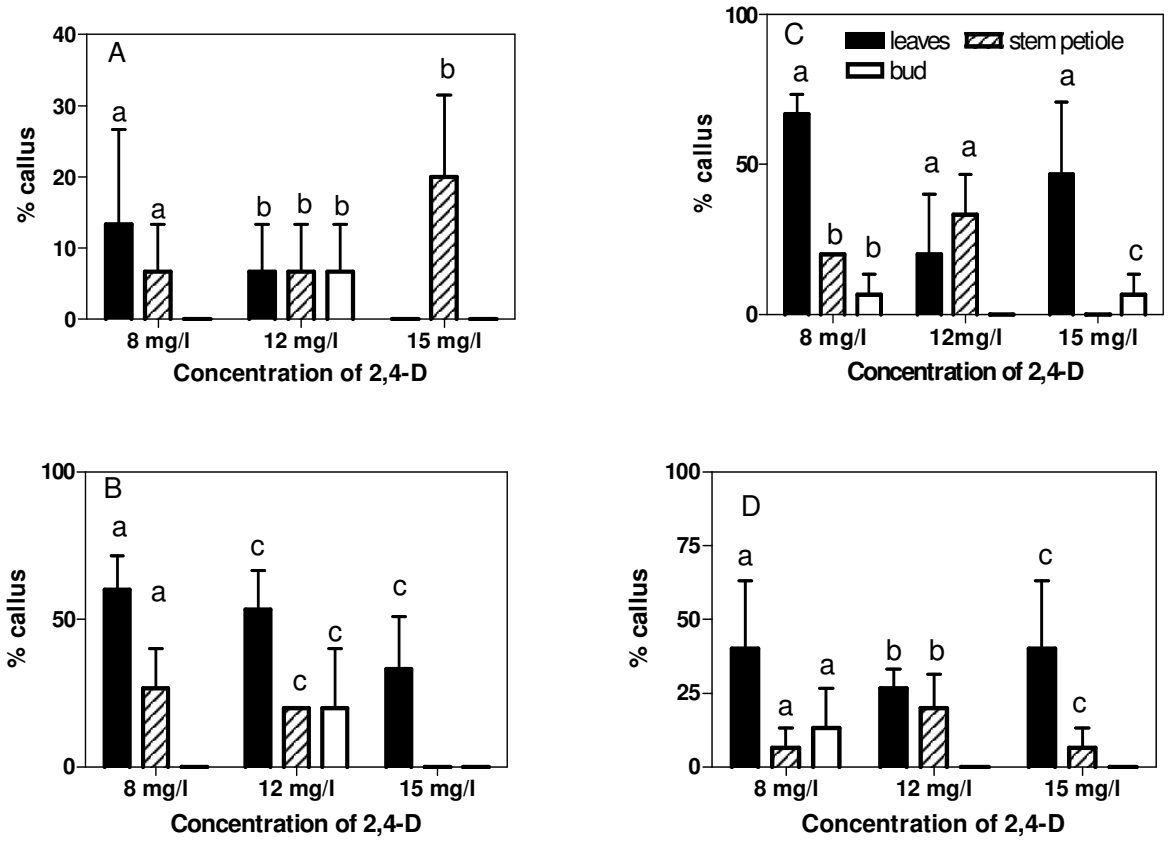

Figure 3. The percentage of callogenic explants of four cassava cultivars. A. Cultivar *Tuaka*; B, cultivar *Afisiafi*; C, cultivar *Afebankye*; cultivar *Doku*. (N = 36; bar = SEM; different letters indicate significant differences).

used (Figure 3a). Generally, the leaf explants were most successful in forming callus and the bark explants were the least. However, the results from *Afisiafi* cultivar show significant increase in the mean percentage of callus formed by the leaf explants when compared with petiole and buds in the 8 mg/l 2,4-D-containing medium (Figure 3b). Effects resulting from the 12 and 15 mg/l 2,4-D were not significantly different among the cultivars. In cultivar *Afebankye* and cultivar *Doku,* the leaf, petiole and buds produced the same amounts of callus upon treatment with the various 2,4-D concentrations (Figure 3c and 3d). Comparing the efficiency in forming callus, the leaf explant, irrespective of the cultivar gave the highest percentage of callus in the tissue culture (Figure 3).

Effect of auxin concentration on callus induction

The three 2,4-D concentrates applied did not significantly affect induction of callus from the explants obtained from *Tuaka* cultivar (Figure 4a). In the *Afisiafi* cultivar, however, the 8 mg/l 2,4-D concentration significantly produced a higher callus from leaf explants than the other concentrations (Figure 4b). There was however, no significant difference ($p < 0.05$) between the three 2,4-D levels for the other explants for the *Afisiafi* cultivar. Results for *Afebanye* and *Doku* cultivar followed a similar trend (Figure 4c and 4d). Bud explant in media containing 12 and 15 mg/l 2,4-D failed to yield callus.

DISCUSSION

The responses to callogenesis and somatic embryogenesis of four cultivars grown in Ghana were investigated. Many works have been carried out on the establishment of callus culture from cassava in different laboratories (Fietosa et al., 2007; Atehnkeng et al., 2005, Schopke et al., 1996). But this is the first time one is done on Ghanaian cassava varieties. Our works carried out on four cultivars grown in Ghana displayed that at 8 mg/l concentration of auxin in culture medium, explants from *Afisiafi* cultivar generated significantly underwent callogenesis when compared with explants from the other three cassava varieties used.

Although, cassava has a generation period of nearly six months, materials for the tissue culture work were produced from 2 to 4 week old plants (Figure 1). Thus, unlike the classical plant breeding which requires pollination and seed development, generation time for

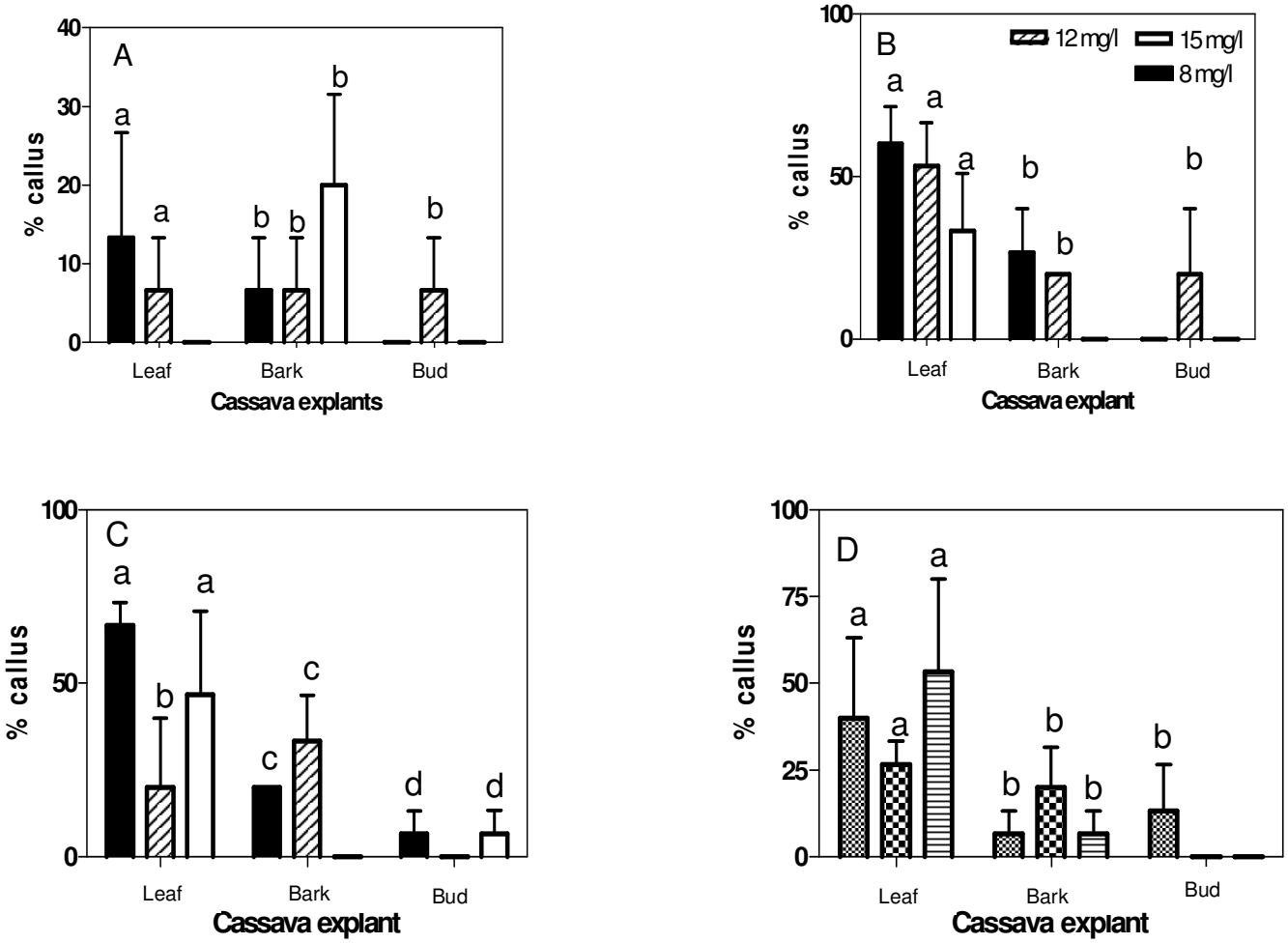

Figure 4. The effect of 2,4-D concentrations on percentage of callogenic explants (callus) induced from cassava explants. The percentage of callus formed from cultivar *Tuaka* (A); cultivar *Afisiafi* (B), cultivar *Afebankye* (C.) and cultivar *Doku* (D) by 8 mg/l (filled bars), 12 mg/l (crossed bars) and 15 mg/l (open bars) are shown in the graph. (N = 36; bar = SEM; different letters indicate significant differences).

new varieties by embryogenic culture via callus culture can be significantly reduced. This also allows precise genetic modification to produce progeny of desirable phenotype.

We have shown in this study that although, the cassava explants used (that is, leaves, stem petiole and buds) can produce callus; there is significant difference in the percentage of callus formed per explants (Figure 3). This broad use of cassava explants for establishing tissue culture with minimal constitution of media provides great opportunity for incorporating the crop in tissue culture for breeding of varieties of desired agronomic importance. This will also provide alternative materials for storing germplasm. Callus is an undifferentiated cell mass produced from differentiated tissues and thus, important material for either directly regenerating plants or vegetative embryogenesis suspension culture. Every differentiated plant tissue is totipotent, but the conditions to de-differentiate them vary from species to species and even from tissue to tissue within same plant (Ezhova, 2003).

Among the three cassava explants used in the experiment, the leaves were the most totipotent with the highest percentage (75% callus at 8 mg/ml) of callus formed in cultures. The leaf parenchyma cells, especially mesophylls, are easily reprogrammed through de-differentiation into undifferentiated cells with characteristics similar to meristematic tissues (Twumasi et al., 200). With the exception of leaf epidermis, leaf cells have thin cell walls which are less lignified (Schädel et al., 2010). These cells are relatively less recalcitrant to differentiation in culture medium due to their high responsiveness to phytohormones (San-José et al., 2010).

One of the four cassava cultivars used in this study, *Afisiafi,* was most callus-inductive at all the three 2,4-D concentrations and explants used (Figure 3) and therefore, indicating high totipotency of the cultivar. Such

variations among explants tissues of some plant species have been well documented. Irrespective of the culture media and cultivar, leaf tissues gave the best yield of callusing. Leaves are known to be most totipotent, capable of becoming de-differentiated tissues (Dhar and Joshi, 2005). In *Zinnia cell cultures,* a perfect model for cell wall synthesis and programmed cell death in plants, mesophyll cells of young leaves are known to be highly totipotent. These cells easily become de-differentiated into undifferentiated cells like mersitematic cell mass which are later differentiated into tracheary elements (Twumasi et al., 2009). Buds are mostly made of meristematic cells which are undifferentiated. Cassava buds as was observed among the four cultivars used in this study, showed the lowest percentage of callus in the media used.

It is concluded from this study that cassava leaf explants are 24.07 and 47.20% higher in the formation of callus when compared with the bud and petiole explants. This trend was similar among cultivars *Afisiafi, Afebankye* and *Doku* tested. It was also realized that 2,4-D concentrations used have no significant differences in the induction of callus using explants from *Tuaka*, *Afebankye* and *Doku* cassava cultivars. However, the 8 mg/l 2,4-D concentration produced the highest number of calli per explants from *Afisiafi* cultivar.

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