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Colchicine-sensitivity Test in Cassava Leaf Lobes and its Effect on Callus and Somatic Embryo Formation

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ABSTRACT

Background: Colchicine acts as a polyploidy inducer but at high concentrations, it causes high cell mortality. To improve the efficiency of colchicine polyploidization in cassava, leaf lobes colchicine-sensitivity tests were carried out and LD_{50} determined at 0.00, 0.05, 0.10, 0.20 and 0.25g/l colchicine concentrations in the varieties *Ankrah, Dagati, Tomfa* and *Tuaka*.

Methods: Colchicine treated leaf lobes were regenerated into callus on either 8 mg/l 2, 4-D or Picloram. The calli were subsequently regenerated into somatic embryos by NAA. LD_{50} of 0.09, 0.11, 0.13 and 0.09 mg/L colchicine concentration were determined for *Ankrah, Dagati, Tomfa* and *Tuaka* respectively in 2, 4-D. Similarly, LD_{50} of 0.12, 0.10, 0.14 and 0.10 mg/L were respectively obtained in Picloram.

Result: In 2, 4-D, *Ankrah* and *Tuaka* were more sensitive to colchicine than *Dagati and Tomfa* whereas in picloram, *Dagati* and *Tuaka* showed more sensitivity. Callus proliferation differed significantly among varieties and influenced by the concentration of colchicine.

Key words: Callus and somatic embryo, Cassava, Colchicine, Induced mutant, Lethal dose.

INTRODUCTION

One of the most useful culinary and industrial crops, cassava (*Manihot esculent*), is prized for its high starch content (Parkes, 2001). Cassava thrives in environments where other crops typically fail, including marginal farmland. Due to the large daily consumption of traditional meals made from the tubers, such as *Fufu, Ampesi and Gari*, the crop is a necessary staple in Ghana (Amenorpe *et al.*, 2006; Baafi and Sarfo-Kantanka, 2008). According to reports, the average person consumes 152.9 kilograms of cassava annually (Baafi and Sarfo-Kantanka, 2008). Additionally, the tubers can be utilized to make fermented starches, dried chips, or animal feed pellets (Prakash, 2018). The paper, cosmetic, textile and pharmaceutical sectors all use cassava starch as one of their primary raw materials (Manyong and Abass, 2007); (Tonukari, 2004). Because bioethanol is a more environmentally friendly fuel than fossil fuels, there is an increase in demand for non-mealy high starch cassava varieties. Therefore, the actors in the cassava value chain obtain appreciable income from cassava farming, processing and selling (Watananonta, 2006).

The genetic variability in cassava is extremely limited because of its vegetative propagation method, which is a significant barrier to cassava improvement. The creation of novel genetic diversity by controlled crossing or mutagenesis is required to improve and make considerable progress in breeding for beneficial traits. Shy flowering is the main barrier to crossover in some areas and there are currently no viable solutions for dealing with non-flowering varieties (Hung *et al.*, 2016). Chromosome doubling in explants and *in vitro* plantlet regeneration using callus and somatic embryos could also help to solve this bottleneck. However, the lack of a good *in vitro* regeneration method, particularly for local cultivars, severely compromises this strategy (Aldemita and Hodges, 1996). Callogenesis is an *in vitro* method that has been used successfully to improve several plants' traits (Guo and Zhang, 2005). To produce calli, leaf lobes, cotyledons and zygotic embryos are typically cultured on a media supplemented with auxins like picloram or 2, 4-dichlorophenoxy acetic acid (2, 4-D) or both (Sofiari, 1996). On NAA medium, such induced calli can be transformed into somatic embryos using developmental processes like those of zygotic embryos. However, because of this system's high genotype dependence, it has not been widely adapted in most crop species. According to Joseph *et al.* (2004), embryogenic calli can potentially be employed to induce mutations.

Somatic embryogenesis in cassava has been achieved mainly from young leaves and shoots meristems (Puonti-

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Kaerlas, 1997; Feitosa *et al.*, 2007). Thus far, it is the only reproducible morphogenic system in cassava which was first reported by Stamp and Henshaw (1982, 1987). Puonti-Kaerlas *et al.*, 1997 and Li *et al.*, 1998 also induced somatic embryos from immature leaf lobes. Comparatively, the leaf lobes technique is more cumbersome than the use of apical meristems. Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos through characteristic embryogenic stages without fusion of gametes (Schumann *et al.*, 1995). Successful long-term, highly regenerable embryogenic suspension cultures (Ondzighi-Assoume *et al.*, 2019) and/or cyclic somatic embryogenic systems (Gautier *et al.*, 2018) have been established for several cassava varieties, allowing for the further development of new varieties through induced mutations.

Cryopreservation, mutation breeding, micropropagation and transformation are all common uses for plant regeneration through somatic embryogenesis (Shmakov and Konstantinov, 2020). *In vitro* colchiploidization of diploid plants to make polyploids is another highly successful method for increasing genetic diversity. Tetraploid plants, for instance, can be created by chemically treating diploid plants with colchicine, oryzalin, *etc.* (Carvalho *et al.*, 2016). Additionally, by mating two ploidy levels together, additional ploidy levels can be produced. The first triploid cassava variety was a hybrid of naturally diploid and artificially induced tetraploid plants (Liu *et al.*, 2021). Colchicine instead of causing complete polyploidy in the plant results in gene alterations in both seed and vegetatively propagated crops (Manzoor *et al.*, 2019). The mode of action of colchicine was exploited to cause morphological changes in eucalyptus and pineapple (Mujib, 2005; Lin *et al.*, 2010). On the local cassava varieties in Ghana, however, there is limited information available regarding colchiploidization and somatic embryogenesis. Colchicine sensitivity testing, determining the Lethal Dose 50 (LD_{50}) in cassava leaf lobes and determining the effectiveness of *in vitro* regeneration of the treated leaf lobes into induced calli and somatic embryos were the goals of this work.

MATERIALS AND METHODS

Plant materials and culture media used

Each cassava stem cutting, which measured 15 cm long, was put in a polythene bag with topsoil and placed in a heat chamber at 37°C for five weeks. *Ankrah, Dagati, Tomfa* and *Tuaka* were the cassava varieties that were utilized. To carry out the experiment, the sprouting stem tips and leaf lobes were cut off. Murashige and Skoog (1962) MS powdered basal medium served as the culture media (Sigma Chemical Company, St. Louis, USA). Based on earlier research, either 8 mg/l of 2, 4-D or 16 mg/l of picloram was added to the MS medium to create callus (Danso and Ford-Lloyd, 2002). Before to being sterilized in an autoclave at 121°C for 15 minutes, all culture media were adjusted to pH 5.8 using 1 M NaOH or 1M HCL with 3.5 g/l phytagel (to facilitate media solidification). In honey jars, 50 milliliters (ml) of medium were used and 15 ml were used in Petri plates. Prior to use, all media were stored at room temperature. Incubation conditions and aseptic manipulations: Under the laminar air flow hood, aseptic manipulations were carried out (Nuaire Biological Safety Cabinet, UK). Scalpels and forceps were sterilized in a Gallenkamp Hotbox oven for two hours at 110°C. All inoculated Petri plates were sealed with parafilm (Pechiney Plastic Packaging, USA) to guard cultures against contamination and desiccation. The cultures were housed in a growth environment with a 16/8h (light/dark) photoperiod and 2300 lux of light provided by white fluorescent tubes. The growth environment was maintained at a constant temperature of 26°C.

Colchicine-Sensitivity test

Leaf lobes from sterilized shoot ends of the *Ankrah, Dagati, Tomfa* and *Tuaka* types were immersed in 10 ml of 0.00 g/l, 0.05 g/l, 0.10 g/l, 0.20 g/l and 0.25 g/l colchicine for an hour in firmly closed glass vials. The orbital shaker for these bottles was set to 6.5 rpm. Treating leaf lobes required three thorough rinses before being added to callus induction media (8 mg/l 2, 4-D or 16 mg/l Picloram; Danso and Ford-Lloyd, 2002) and incubated for 21 days at 21°C in full darkness. The size and color of the callus development were assessed after the incubation period of 21 days. The factorial design of the experiment was entirely random in its construction. The variables examined were (four cassava varieties) x (four concentrations of colchicine).

Embryogenesis of induced callus

The calli were moved to MS media that additionally included 30 g/l of sucrose, 100 mg/l of myo-inositol, 0.01 mg/l of naphthalene acetic acid (NAA) and 0.1 mg/l of 6-benzylaminopurine (BAP). Before the medium was autoclaved at 121°C for 15 minutes at 15 psi, Phytagel was added and the pH was adjusted to 5.8. The cultures were then kept in a growth environment with white fluorescent tubes (T5 fluorescent fitting, UK) emitting light with a 3000-lux intensity at a temperature of 21°C and a photoperiod of 16/8 hours (light/dark). The cultures were examined for somatic embryo development after two weeks and the quantity of somatic embryos produced per clump was noted. Three callus clumps were included in each of the three replications of each treatment. The statistical analysis was completed using Genstat software, version 15. At the 5% confidence level, the least significant difference (LSD) was employed to distinguish between significant ANOVA.

RESULTS AND DISCUSSION

Colchicine-sensitivity curve of cassava leaf lobes incubated on 2, 4-D or Picloram

After 21 days of dark incubation, callus production in colchicine-treated leaf lobes of four different varieties of cassava on 2, 4-D or picloram supplemented media was assessed. On 2, 4-D-supplemented, non-treated (control) explants recorded the highest proportion of callus formation

compared to explants that had received colchicine (Fig 1). When the concentration of colchicine was increased from 0.00% to 0. 25%, the number of calli clumps significantly decreased ($0.8 \leq R2 \geq 0.9$). In a similar vein, the proportion of callus formation in leaf lobes treated with colchicine and inoculated on media supplemented with picloram reduced as colchicine concentration increased from 0.00% to 0. 25% ($0.5 \leq R2 \geq 0.8$) (Fig 1). As colchicine concentration increased, callus formation decreased in colchicine-treated leaf lobes but higher in untreated (control) leaf lobes. Due to its antimitotic activity, colchicine has been widely used to induce artificial polyploidization in plants (Germana, 2012).

Due to its capacity to block the separation of divided nuclei during the cell cycle during anaphase, colchicine raises the ploidy levels of mitotic cells. This might result in the creation of polyploid plants, which improves the genetic diversity of crops. By increasing the number of dominant alleles or reducing the negative effects caused by recessive alleles, polyploidization in plants increases adaptability to environmental pressures or changes (Van de Peer *et al.*, 2021; Soltis *et al.*, 2015). Asexual reproduction, heterosis and gene redundancy (caused by gene duplication) are further benefits of polyploidy in plants (in certain cases the facilitation of reproduction through self-fertilization or asexual means). Gene redundancy protects polyploids from the harmful effects of mutations, whereas heterosis makes polyploids stronger than their diploid ancestors (Van de Peer *et al.*, 2021; Chevasco, 2012; Zhang, 2008). The decrease in callus production and/or size seen in this study because of increased colchicine concentrations suggests that embryogenic tissues have a hermetic response to colchicine, which could explain for the decrease in callus clump size.

LD_{50} of colchicine-treated cassava leaf lobes

Fig 2 displays the colchicine-treated leaf lobes of four types of cassava that were inoculated on 2, 4-D, or picloram-supplemented media. The estimated lethal dose (LD50) for colchicine-treated leaf lobes of the *Ankrah, Dagati, Tomfa* and *Tuaka* types were 0.09, 0.11, 0.13 and 0.09% mg/L on 2, 4-D media and 0.12, 0.1, 0.14 and 0.1% mg/L on picloram medium, respectively. The sensitivity based on the estimated LD_{50} for colchicine-treated leaf lobes of the four varieties *Ankrah* (0.09),

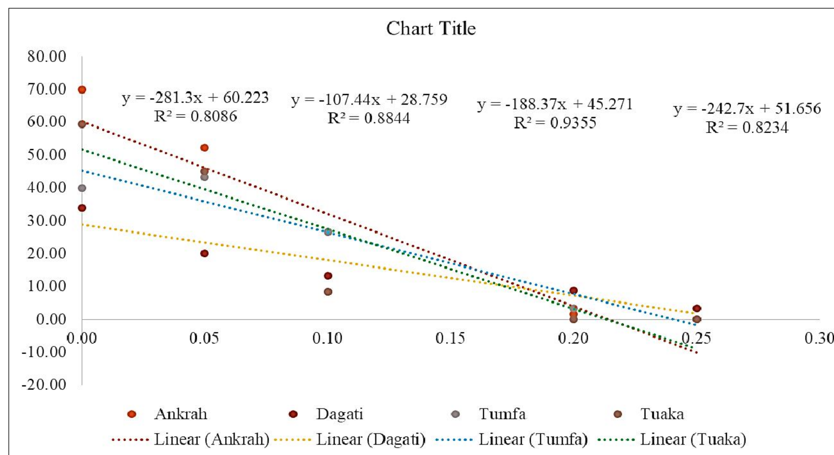

Fig 1: Calli developed (%) at varying concentration of colchicine on 2, 4-D medium.

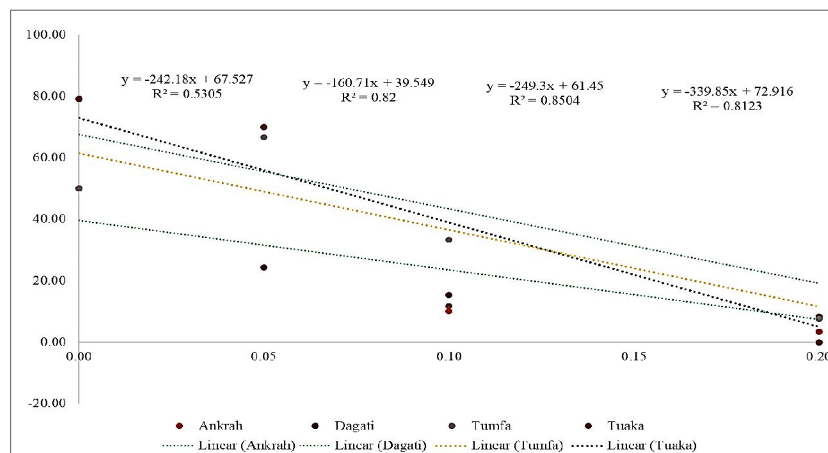

Fig 2: Calli developed (%) at varying concentration of colchicine on picloram medium.

Dagati (0.11), *Tomfa* (0.13) and *Tuaka* (0.09) % mg/L of colchicine treatment on 2, 4-D and treatment in picloram medium as *Ankrah* (0.12), *Dagati* (0.1), *Tomfa* (0.14) and *Tuaka* (0.01) % mg/L of colchicine, indicated that the LD_{50} for colchicine depends on variety as *Ankrah* and *Tuaka* were more sensitive to colchicine than the other cassava varieties.

Most plant breeders prefer applying LD_{50} as an effective acute dose for mass sample mutagenesis to achieve average survival rates. However, in practice it is better to mass irradiate at a bit higher dose than the LD_{50} for discovery of more useful mutants. A single acute dose range of LD_{50} (±10%), or an acute dose resulting in 20% survival of treated material could cause effective mutations (Amenorpe *et al.*, 2010; Heinze and Schmidt 1995).

Number of days to callus formation in colchicine-treated leaf lobes

The number of days taken for colchicine-treated leaf lobes to develop callus recorded on 2, 4-D and picloram supplemented media (Table 1). The colchicine treatment had significant ($P \leq 0.05$) effect on the number of days to callus formation in leaf lobe explants of the four cassava varieties. Higher concentrations of colchicine delayed callus formation in all four cassava varieties. However, differences were observed in variety response to callus formation from colchicine-treated leaf lobes cultured on picloram-amended medium. Callus formation in the varieties *Dagati* and *Tomfa* delayed longer with increasing concentration of colchicine until 0.2 g/l compared to *Ankrah* and *Tuaka* (Table 1). *Ankrah* produced a callus in 0.05 mg/L concentration of colchicine within 8 days whilst *Dagati* took 13 days. Similarly, at 0.1 and 0.2 mg/L concentrations of colchicine, *Tuaka* and *Ankrah* produced calli earlier compared to *Dagati* and *Tomfa*.

The number of days taken for colchicine-treated leaf lobes to develop callus on 2, 4-D was delayed by higher concentration of colchicine (Table 2). The colchicine treatment had significant ($P \leq 0.05$) effect on the number of days for leaf lobes to develop callus. As the concentration of colchicine increased, leaf-lobes took longer days to form callus. *Ankrah* produced a callus in 0.05 mg/L concentration of colchicine within 9 days whilst *Dagati* took 14 days. Similarly, at 0.1 and 0.2 mg/L concentrations of colchicine, *Dagati* and *Tomfa* had relatively lower number of days to callus production compared to the other varieties. Leaf lobes of varieties *Ankrah, Dagati, Tomfa* and *Tuaka* took different days (17, 19, 20 and 18 days respectively) to develop callus on picloram medium. The delay in callus formation of genotypes under varying concentration of colchicine was likely due to the genoype. Tissue-type sensitivity to colchicine has been observed in the different response of apical and lateral explants to colchicine treatments, showing a diversity in antimitotic sensitivity (Manzoor *et al.*, 2019; Carvalho *et al.*, 2016). This emphasizes the need for radiosensitivity test to be done on the type of explant or genotype used prior to large scale treatment for mutation induction.

According to Snehal and Madhukar (2012), higher concentrations of colchicine affect callus growth negatively, therefore causing delay in callus emergence in cassava leaf lobes. Colchicine at higher concentration may adversely restrict the mitotic process essential for callus formation because of the destruction of some of the cellular organelles (Manzoor *et al.*, 2019). This suggests that faster callus emergence can be moderated by controlling the concentration of colchicine. However, lowering the concentration of colchicine is detrimental to discovery of new mutants because the mutation frequency rate is seriously reduced.

Somatic embryogenesis

Somatic embryogenesis has become an important technique for plant regeneration and production of totipotent tissues in cassava. Calli cultures were assessed for somatic embryo

Table 1: Days to callus formation with increasing concentration of colchicine on picloram media.

Variety	Concentration of colchicine (g/l)					Mean
	0.00	0.05	0.1	0.2	0.25	
Ankrah	9.00^{ab}	8.00^{a}	20.00^{ef}	19.00^{def}	30.00^{h}	17.2
Dagati	9.00^{ab}	13.00^{abcd}	21.00^{efg}	23.00^{fg}	30.00^{h}	19.2
Tumfa	9.00^{ab}	11.00^{abc}	23.00^{fg}	26.00^{gh}	30.00^{h}	19.8
Tuaka	9.00^{ab}	9.67^{ab}	19.33^{cde}	19.33^{def}	30.00^{h}	17.5

Values with same superscripts are not significantly different at $P \leq 0.05$.

Table 2: Days to callus formation on 2, 4-D media by colchicine treated leaf lobes.

Variety	Concentration of Colchicine (g/l)					Mean
	0.00	0.05	0.1	0.2	0.25	
Ankrah	9.00^{ab}	10.00^{abc}	26.00^{ghi}	22.00^{f}	30.00^{i}	7.92
Dagati	9.00^{ab}	14.00^{bcde}	25.00^{ghi}	27.00^{hi}	30.00^{i}	7.60
Tumfa	10.00^{abcd}	11.00^{abcd}	26.00^{ghi}	17.00^{cdef}	30.00^{i}	7.36
Tuaka	9.00^{ab}	11.00^{abcd}	26.00^{ghi}	20.00^{efgh}	30.00^{i}	7.36

Values with same superscripts are not significantly different at $P \leq 0.05$.

formation and the number of somatic embryos produced per clump was recorded after two weeks in light. Fig 3 shows colchicine treated leaf lobes of *Ankrah* had developed calli on 2, 4-D and embryo on NAA in two weeks. Similarly, the colchicine treated leaf lobes of *Tuaka* also developed calli on picloram and embryo on NAA. It was observed that the number of somatic embryos produced per clump was independent of the size of the calli clump formed. A callus clump may be small yet contained smaller colchipoid cells with higher regeneration rate into embryo depending on the genotypes. Moreover, varieties may respond differently to auxin and cytokinin in the NAA media.

Colchicine treated leaf lobes of *Ankrah* developed calli clump on 2, 4-D and embryo on NAA in two weeks. Similarly, the colchicine treated leaf lobes of *Tuaka* also developed calli on picloram and embryo on NAA media in the same period, but their sizes differ (Fig 4). This was possible because NAA media has both auxins combined with cytokinins. Auxin decides the pace at which callus is going to develop further. It initiates somatic embryogenesis by inducing stress response in plant cells while the cytokine induces cell division in the explants (Teixeira da Silva and Malabadi, 2005). Cytokinin influences cell-to-cell auxin transport by modification of expression of several auxin

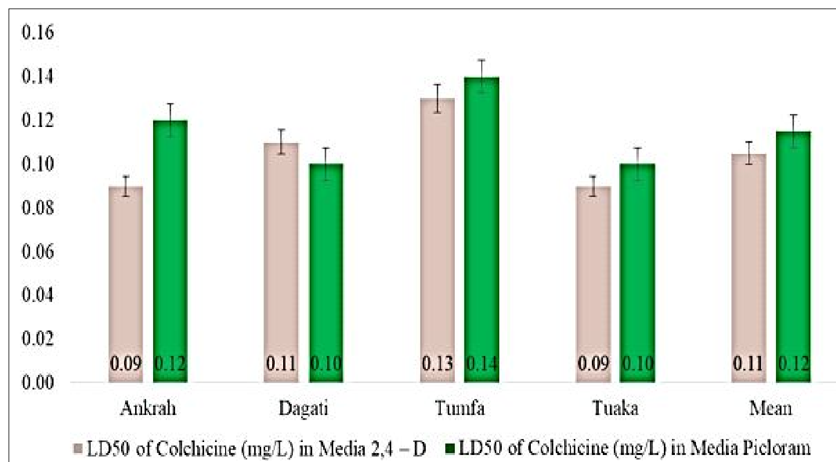

Fig 3: Sensitivity of genotypes to colchicine treatment on 2, 4-D and picloram media.

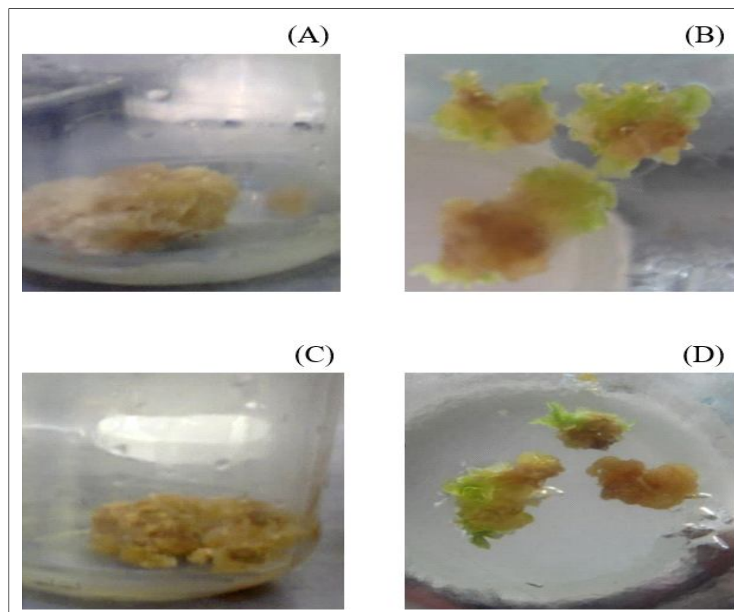

Fig 4: Colchicine treated leaf lobes of *Ankrah* developed calli on 2, 4-D (A) and embryos on NAA (B). Colchicine treated leaf lobes of *Tuaka* produced calli on picloram (C) and embryos on NAA (D).

transport components and thus modulates auxin distribution important for regulation of activity and size of the root meristem (Swarup *et al*., 2019; Rùžièka *et al*., 2009). It was observed that tight calli with shoot organic potentiality failed to develop into somatic embryos but only loose calli without shoot organic potentiality developed somatic embryos.

The mutagenic treatments affected the days to somatic embryogenesis as was seen in colchicine treatments compared to the controls. This result undoubtedly confirmed colchicine as an anti-mitotic agent, which suppressed mitotic division and hence inhibited somatic embryogenesis. On the other hand, Somatic embryo formation is influenced by several factors such as explant, growth hormones and environmental factors (Bogdanović *et al*., 2021). Of these factors, growth hormones are the most pronounced. In this study, MS basal medium supplemented with 8 mg/l 2, 4-D led to early somatic embryo development as compared to 16 mg/l picloram.

CONCLUSION

The study revealed that callus proliferation differs significantly among varieties and dependent upon the concentration of colchicine. The delay in callus formation although colchicine acts as a polyploidy inducer in plants, it exhibits toxic effects at high concentrations, leading to high mortality in treated tissues/plants. Thus, to optimize the use of colchicine for induction of polyploidization using *in vitro* tissues, radiosensitivity tests must be carried out to determine the lethal dose (LD50) for different crops. The LD_{50} for colchicine-treated leaf lobes of *Ankrah, Dagati, Tomfa* and *Tuaka* varieties were estimated to be respectively, 0.09, 0.11, 0.13 and 0.09% mg/L of colchicine on 2, 4-D medium and 0.12, 0.1, 0.14 and 0.1% mg/L of colchicine respectively on picloram medium.

A significant decrease in callus production was observed in this study. This observation was speculated to be due to considerable tissue damage known to be caused by high concentrations of colchicine. In addition, this study proved that callus induction ability was greatly influenced by the variety used. The leaf lobes of *Ankrah* and *Tuaka* were more sensitive to colchicine than the rest. This demonstrates that different varieties respond differently to callus formation. Some varieties respond to colchicine earlier than others, a difference that appears to be genetically controlled. The delay in callus formation in the different cassava varieties under varying concentrations of colchicine was also observed to depend on biotype.

It was observed that the number of somatic embryos produced per clump was independent of the size of the calli clump formed because calli clump may be small but contained more live cells than in larger clumps for regeneration into embryos. It was further observed that tight calli with shoot organic potentiality failed to develop into somatic embryos but only loose calli without shoot organic potentiality developed somatic embryos. The induction of calli with colchicine had an anti-mitotic effect on calli clumps and suppressed the mitotic division during somatic embryogenesis than controls. Another factor is genotypes may respond differently to auxin and cytokinin in the NAA media.

Conflict of interest: None.

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