ISSN: 3064-9846
Research Article

Journal of Agricultural, Earth and Environmental Sciences

Promotion of Indirect Somatic Embryogenesis of *Coffea Arabica* var Geisha and Improved N39-6 Hybrid through Auxin-Cytokinin Coordination

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Submitted: 16 December 2024 **Accepted:** 24 December 2024 **Published:** 02 January 2025

https://doi.org/10.63620/MKJAEES.2025.1068

Citation: *Ramadhani, F. J., Mtenga, D., Mwaipopo, R. E., Aman, N. M., Mbwambo, S., & Kilambo, D. (2025). Promotion of Indirect Somatic Embryogenesis of Coffea Arabica var Geisha and Improved N39-6 Hybrid through Auxin-Cytokinin Coordination. J of Agri Earth & Environmental Sciences, 4(1), 01-08.*

Abstract

Somatic embryogenesis has been used to produce different coffee varieties using auxin-cytokinin interaction. The procedure has been used to multiply millions of coffee plantlets worldwide although its success relies on a particular genotype. Despite the significance of auxin-cytokinin interaction in Coffea arabica micropropagation, little is known about their ability to promote callus-induced plantlets from C. arabica var Geisha (Geisha) and improved N39-6 hybrid in vitro. Thus, this study evaluated the potency of auxin-cytokinin interaction using Geisha and N39-6 coffee leaf explants. Geisha and N39-6 leaves were sterilized,excised into small fragments of around 1 cm2. These fragments were then cultured on Murashige and Skoog (MS) with different concentration of 2,4-D, 2 iP, BAP, and sucrose to activate indirect somatic embryogenesis. Media with 0.5 mg/L 2,4-D and 2 mg/L 2iP promoted callus cell formation around the leaf regions, and the percentage of the area covered by callus cells was significantly higher in Geisha scoring 76-100% (46.3) compared to 76-100% (31.3) of N39-6 within 30 days. Then, callus-formed explants transferred on 1 mg/L 2,4-D and 4 mg/L BAP developed into friable and compact calli on Geisha and N39-6 explants respectively. However, N39-6 calli was heavier (172g) unlike Geisha (163.5g). Friable and compact calli established on the media supplemented with BAP 1.125 mg/L promoted the formation of globular, torpedo, and heart-shaped embryos in both explants. The MS media containing 1.125 mg/L BAP and 0.01 mg/L Biotin advanced to the cotyledonary stage and produced roots in Geisha and N39-6 somatic embryos. The fully formed plantlets adapted well to the ex vivo environment. Our findings indicate that this methodology can be utilized to create plantlets for several genotypes. Nevertheless, to ensure that the majority of hybrid varieties can be cultivated in vitro, more research is required.

Keywords: Indirect Somatic Embryogenesis, Auxin, Cytokinin, *Coffea arabica*, Geisha Coffee, and Improved Coffee Variety

Introduction

Coffee is a perennial crop with the two predominant varieties of *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta), mostly consumed as a beverage. However, coffee's functions have been extended to pharmaceuticals and cosmetics industries using its bioactive molecules for therapeutic and health advantages [1]. Leveraging coffee sustainability and productivity, the Tanzania Coffee Research Institute (TaCRI) has achieved a significant milestone by releasing over ten improved commercial N39 Coffea arabica hybrids (N39-1 to N39-12). These hybrids have economic benefits such as high yields, exceptional cup quality, and resistance to *Colletotrichum kahawae* (coffee berry disease agent) and *Hemileia vastatrix* (coffee leaf rust agent) [2-5]. The N39-6 is the bourbon tall Arabica hybrid variety, with an average of 2,891 kg/ha, a pleasant aroma, bean size 77 (AA+A), and a good cup of class 4+. Efforts have been made to adopt these new technologies for farmers and coffee stakeholders to increase the productivity and sustainability of the coffee industry in Tanzania. Coffee seedlings have been propagated using several methods such as seeds, clones, and grafting globally [6-8]. However, plans for the

sustainability of coffee production using the mentioned methods might encounter difficulties like climate change, and insufficient capital and manpower in the future [9, 10]. Thus, to ensure constant availability of coffee seedlings the development of in vitro micropropagation protocol is necessary. A previous study from TaCRI looked at how some of the improved N39 varieties could respond under tissue culture environments and discovered that micropropagation of N39-1 and N39-5 improved hybrids from leaf explants regenerate plantlets via somatic embryogenesis [11]. Therefore, testing and standardizing the potency of this approach for the micropropagation of different N39 coffee hybrids is an inescapable requirement.

Coffea arabica var Geisha has originated in Ethiopia since the 1930s, this coffee variety was subsequently exported to Tanzania and in 1953 expanded to the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Central America. After that, the Geisha crossed into Yemen and, more precisely, Panama in South America. By the 1960s, it was made available all over Panama due to its resistance to coffee rust, but not much was planted as the plant's branches were weak [12, 13]. Although Geisha is a native Ethiopian coffee, Panama's selection gained more popularity in the world coffee market ranking as the best coffee based on the unique (distinctive) cup quality attributes [13]. Despite their vulnerability to Coffee Berry Disease (CBD), Geisha possess other remarkable features like large bean size and resistance to leaf rust race II agent [14]. Based on these properties of Geisha coffee, coffee stakeholders requested TaCRI to produce a significant amount of true-to-type geisha seedlings in Lyamungo, Tanzania considering that Geisha trees located at TaCRI resemble the Panama genotype. For several years, the multiplication of coffee plantlets has been done using horticulture procedures such as grafting, seedlings, and cuttings [8, 15]. However, the minimal number of geisha mother plants in our country-imposed challenges to regenerate true-to-type geisha plantlets using horticultural techniques. Therefore, it is necessary to develop a working protocol for geisha micropropagation *in vitro*.

Usually, plants regenerate new adult plantlets from a disorderly collection of cells known as callus in conditioned environments such as wounds and hormonal interaction via somatic embryogenesis [16-18]. This non-sexual process causes somatic cells to release embryos required for complete plant regeneration [19]. Somatic embryos that can be created directly from somatic cells are referred to as direct somatic embryogenesis (DSE), while those obtained from callus induction followed by somatic embryo maturation are referred to as indirect somatic embryogenesis (IDSE) [20]. These two methods are archived via the interaction of plant growth regulators mainly auxin and cytokinin [21-23]. Specifically in coffee, different studies have shown that the interplay of these two hormones aids in the preparation of micropropagation methods for coffee multiplication in vitro [24, 25]. Furthermore, recent research employing RITA® bioreactors with a temporary immersion system has demonstrated the potential micropropagation relevance of the auxin-cytokinin interaction in the bulk generation of Coffea arabica plantlets [26, 27]. Although somatic embryogenesis showed promising micropropagation results in different coffee species, the success of this method depends on the environment, source of the mother plants, and genotype of a particular variety [28]. Thus, it is mandatory to unlock desirable concentrations of auxin-cytokinin for developing regenerative tissue culture protocols.

The potential role of auxin and cytokinin in coffee plant regeneration via somatic embryogenesis has been demonstrated in different publications [26, 29, 30]. Despite the significance of auxin-cytokinin interaction in *Coffea arabica* micropropagation, little is known about their ability to promote callus-induced plantlets from C. arabica var Geisha (Geisha) and improved N39-6 hybrid *in vitro*. Thus, the current study checked the potency of this hormonal interaction to develop a working protocol for the multiplication of Geisha and N39-6 plant lefts from leaf explants *in vitro*.

Material and Methods

The experiments were conducted in the Tanzania Coffee Research Institute (TaCRI) plant tissue culture laboratory in Lyamungu, Hai district, Kilimanjaro region, Tanzania. Geisha and N39-6 leaves were collected from the Geisha plot and clonal garden respectively at Lyamungu.

Plant Materials Preparation

Before the culturing process, Geisha and N39-6 leaves were washed with running tap water and detergent for five minutes and then soaked in 70% (v/v) ethanol for 30 seconds and immersed in a 3.5% (v/v) calcium hypochlorite solution for 30 minutes. Afterward, they were rinsed three times with sterile distilled water under a sterile environment. The sterile leaves were prepared by removing the margins and midribs then excised into 1 cm2 pieces and inoculated on the media [31]. Four different media combinations were formulated with half-strength Murashige & Skoog, 1962 (MS) with other components like plant growth hormones; (2,4-dichloro phenoxy acetic acid (2,4-D), benzyl amino purine (BAP)), biotin, sucrose, agar, phytagel, thiamine (5.0 mg/L), myo-inositol (50 mg/L), nicotinic acid (0.5 mg/L), pyridoxine HCL (0.5 mg/L), 2iP (2.0 mg/L) casein hydrolysate (100 mg/L), 2 mg/L of 2iP, malt extract (400 mg/L), 2,4-D (0.5 mg/L), AIB (1.0 mg/L), sucrose (30 g/L) and gelrite (2.5 g/L) as a solidifying agent were used as explained before (Fig.1) [11]. All the media combinations were sterilized at 121°C for 20 minutes post 5.8 pH adjustment and agar addition [32]. Stage 1, 2, and 3 culture vessels were placed in aluminium boxes with lids and stage 4 bottles were maintained under cool white fluorescent tube lights at 25±1°C.

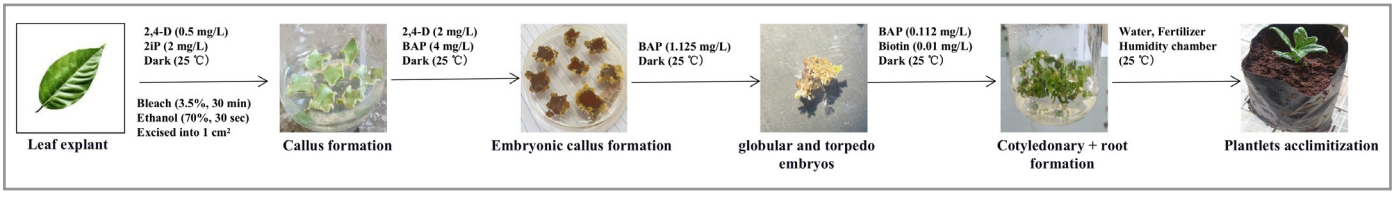

Figure 1: Summarized somatic embryogenesis procedures: A series of events showing cytokinin and auxin interplay concentrations and conditions used on each stage of *in vitro* micropropagation of Geisha and N39-6.

Callus Induction

Callus formation was induced by the wounding method [33]. The 1 cm2 pieces of sterile leaf explants with the adaxial surface facing upward were inoculated in T1 media containing 30 ml of MS medium supplemented with 2,4-D (0.5 mg/L), 2iP (2 mg/L), sucrose (30 g/L), and gelrite (2.5 g/L) at 25±1°C in the dark place. Three days after inoculation we checked the contamination status and weighed the fresh explants. Thirty days later, we assessed the callus initiation status by calculating the percentage of the leaf area covered by callus cells (% LACC) using four ranges of 0-25%, 26- 50%, 51-75%, and 76-100% respectively [31].

Embryonic Callus Stimulation

30 days later, callus-induced explants were transferred to T2 media composed with MS media, 2,4-D (1 mg/L), BAP (4 mg/L), gelrite (2.5 g/L), and sucrose (30 g/L) to promote indirect embryonic callus. All the culture bottles were placed in the dark using aluminium boxes with lids at 25±1°C for 4 months. Subculture of the embryonic callus was performed after every three months. The weight of each bottle containing explants and the bottle with media only (control) were weighed six months after being transferred to T2 media using an analytical scale.

Somatic Embryo Maturation

The friable and yellowish callus were transferred to T3 media with MS media, BAP (1.25 mg/L), gelrite (4 g/L), and sucrose (30 g/L) in the dark area using aluminium boxes with lids at 25±1°C for 3 months onwards.

Cotyledonary and Root Formation (Germination)

Torpedo and heart-shaped embryos were cultured in the T4 media containing MS media, BAP (0.112 mg/L), Biotin (0.01 mg/L), sucrose (10 g/L), and gelrite (4 g/L) to influence germination of the embryos. All the magenta bottles containing embryos were exposed to full-time light provided by cool white fluorescent tubes at 25±1°C.

Acclimatization of Plantlets

Callus induced plantlets were separated from MS solid media and transferred on the surface of the magenta containers containing sterilized cocopeats media for two weeks. In humidified chambers at 25±1 °C, the plants were planted two weeks later in cocopeat

Statistical Analysis

The mean ± standard deviation of three separate experiments was used to show all data. Student's t-test and PASW Statistics software version 18 (SPSS, Inc.) were used for statistical analysis. The differences between means were compared using one-way analysis of variance (ANOVA) and Tukey's test for post-hoc analysis. $P < 0.05$ was regarded as a statistically significant value.

Results

Callus induction

The excised explants on T1 media induced callogenesis on both Geisha and N39-6 explants by promoting whitish swelling on Geisha and N39-6 leaf explants within one month (Fig. 2A and 2B). Also, there is a significant increase in the percentage of areas covered by callus ($p < 0.001$) for Geisha and N39-6 explants based on callus coverage scores of 0-25% (8.8 and 12.5), 26-50% (13.8 and 22.5), 51-75% (31.3 and 33.8) and 76-100% (46.3 and 31.3) respectively (Fig. 2C). This result showed that callogenesis is more active on Geisha explants than N39-6 explants.

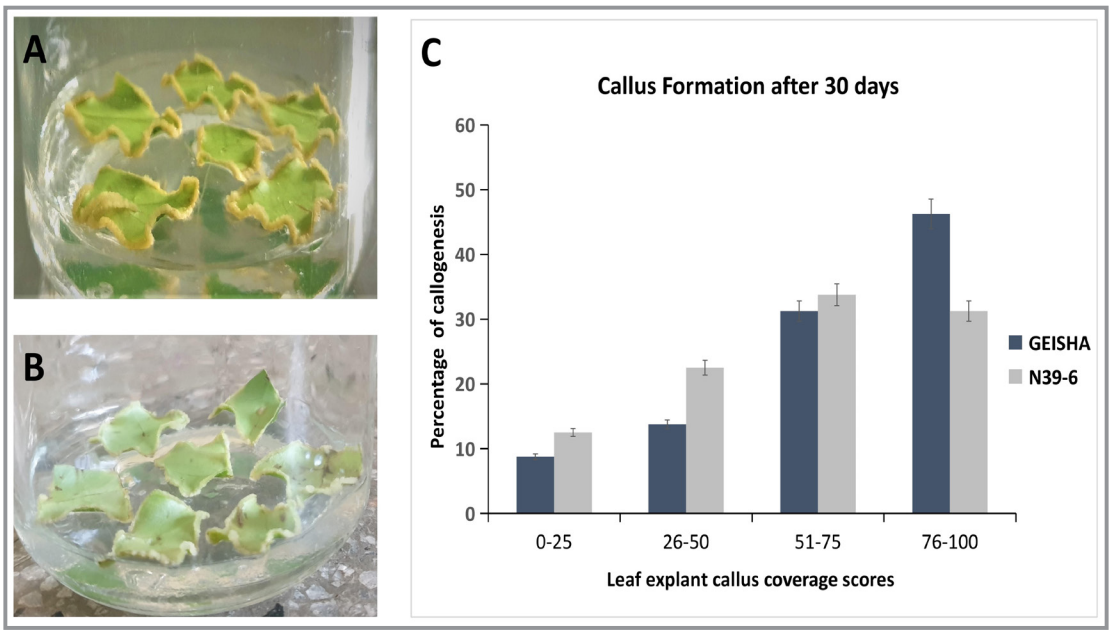

Figure 2: Callus formation after 30 days on MS supplemented with auxin and cytokinin: T1 media produced whitish swelling around excised leaf explants for Geisha (A) and N39-6 (B). Chart graph showing the percentage of callus formation scores as indicated (C).

Embryonic Callus Development

Geisha and N39-6 callus-induced explants were transferred to T2 media to advance in embryonic callus. T2 media transformed the callus to a friable and compact embryonic callus on Geisha and N39-6 explants after 6 months respectively (Fig. 3A and 3B). This data showed a significant increase in callus weight from control (160.28g) to Geisha explants (163.54g) and N39-6 explants (172.36g) (Fig. 3C).

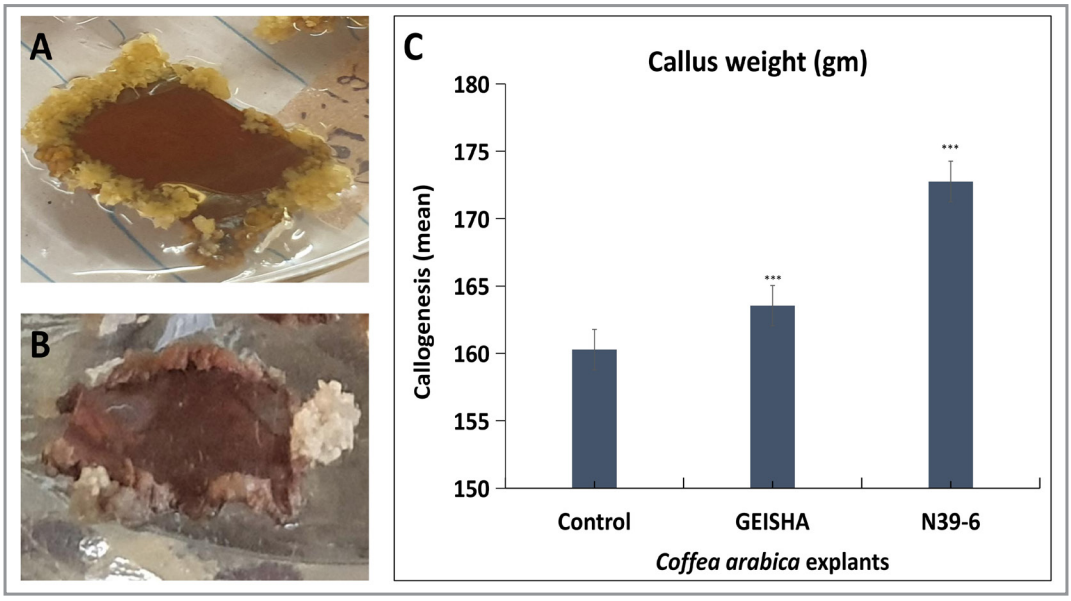

Figure 3: Somatic callus formation on Geisha and N39-6 explants cultured on auxin and cytokinin for more than 4 months; The results show the friable callus on Geisha explant (A) and N39-6 explant (B). Average callus weight of the explants on T2 for Geisha and N39-6 leaf explants after six months (C). Data are presented as the mean ± SD where *** $P < 0.001$ is considered a significant level; Control: media without hormones, Geisha and N39-6 hybrid.

Maturation of Somatic Embryos

Somatic embryos were generated from friable and compact calli on T3 media for Geisha and N39-6 respectively. The results showed that T3 media enhanced different stages of embryo maturation. The formation of the globular and torpedo embryo was observed in both Geisha and N39-6 embryonic callus and a few heart-shaped embryos on Geisha. However, Geisha transformation was faster and more active within 3 months in contrast with N39-6 explants that prolonged for more than 4 months (Fig 4A and 5A).

Plantlets Germination

The results demonstrated that T4 media with a low concentration of BAP and biotin enhanced the majority of heart-shaped embryos, cotyledons, and root formation for both Geisha and N39-6 somatic embryos (Fig 4B and 5B). At different periods, the cotyledons germinated into full plants (Fig 4C and 5C). The plantlets with 3 cm roots were transferred to an acclimatization facility for a gradual hardening process. As seen in Fig 4D and 5D, Plantlets transferred to cocopeats media survived an external environment. Our gradual hardening process responded well to the external conditions. Eight months later, the Geisha and N39-6 plantlets whose roots measured 15 cm and 16 cm respectively were transferred to plastic bags (Fig 4E and 5E) to allow the maturity of roots and leaves before moving them to normal coffee seedlings nursery.

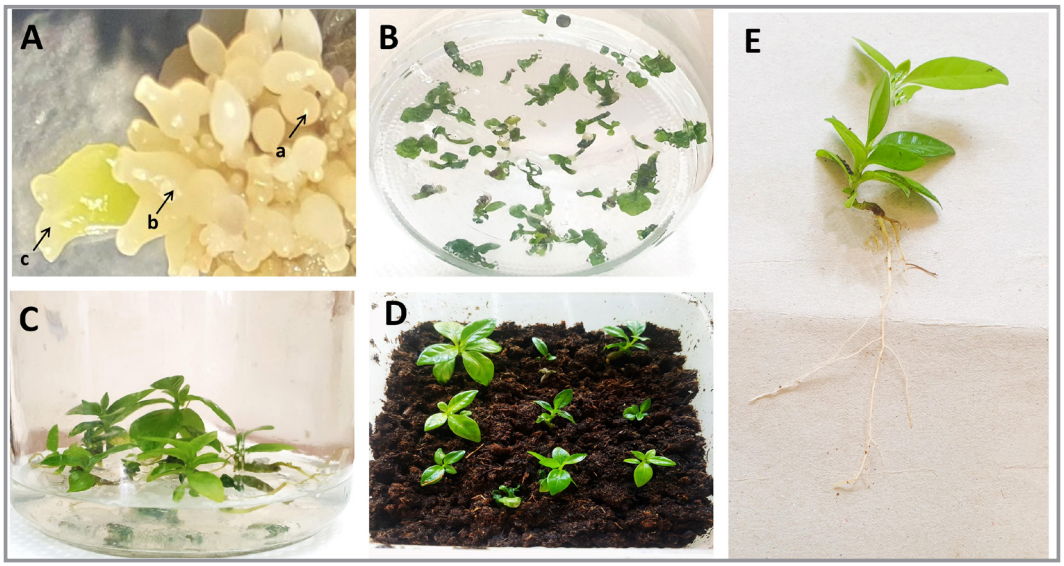

Figure 4: Proliferation of somatic embryos to mature Geisha plantlets. (A) Different stages of somatic embryos on T3 media from friable callus; arrows show a-globular, b-torpedo, and c-heart shaped embryos (B) The cotyledonary stage for Geisha

somatic embryos and (C) The rooting stage for Geisha on T4 media (Biotin (0.01 mg/L) and BAP (0.112 mg/L)). (D) Transferred Geisha plantlets in cocopeat media and gradually nursed to adopt external conditions in a controlled acclimatization house. (E) Eight months later Geisha plantlets with complete roots (15 cm) were moved to plastic bags.

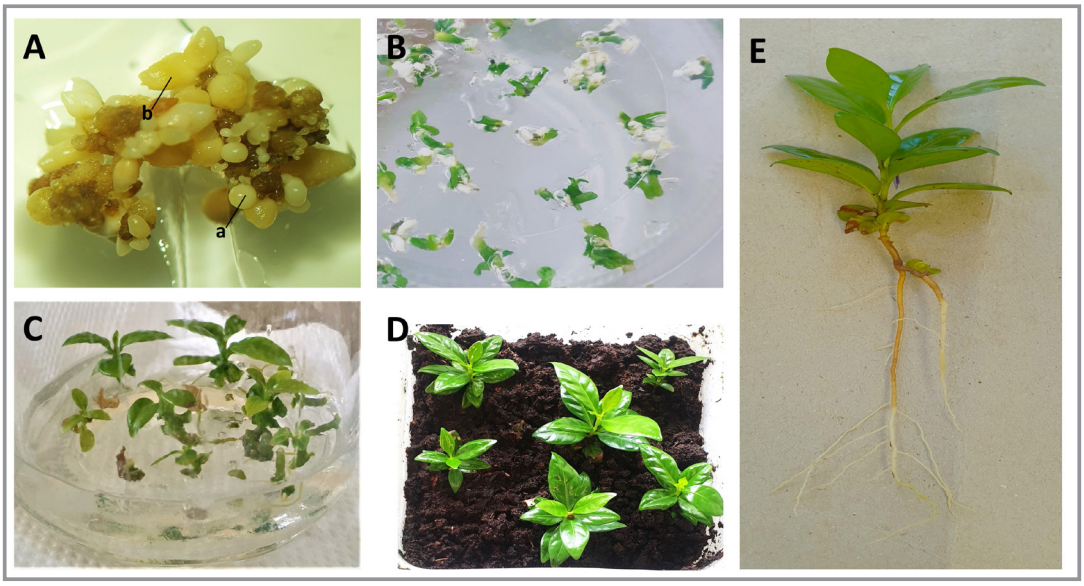

Figure 5: Proliferation of somatic embryos to mature N39-6 plantlets: (A) Different stages of somatic embryos on T3 media from friable callus; arrows show a-globular, b-torpedo embryos. (B)The cotyledonary stage for N39-6 somatic embryos and (C). The rooting stage for N39-6 cotyledons on T4 media (Biotin (0.01 mg/L) and BAP (0.112 mg/L)). (D) Transferred N39-6 plantlets in cocopeat media and gradually nursed to adopt external conditions in a controlled acclimatization house. (E) Eight months later N39-6 plantlets with complete roots (16 cm) were transferred to plastic bags.

Discussion

In the current study, we evaluated the potency of auxin-cytokinin interaction for somatic embryogenesis of Geisha and N39-6 improved hybrids from coffee leaf explants. Since the interaction between cytokinin and auxin is essential for callus formation, their antagonistic role led to the regeneration of new plants via somatic embryogenesis [16]. The potential role of auxin-cytokinin interaction for plant regeneration has been reported in different coffee varieties [21, 25, 27]. Callus formation is the main step for indirect somatic embryogenesis [34]. This paper showed that Geisha and N39-6 leaf explants cultured on 2,4 D and 2iP promoted callogenesis, this interaction caused a significant increase in the percentage of the area covered by callus on both explants as seen in Fig. 2. However, Geisha callus coverage areas were higher than N39-6 confirming that auxin-cytokinin interplay is a genotype dependant [35]. Similar results were documented in a recent study by Alves et al., 2018 that Mundo Nova cultivar (*Coffea arabica*) explants established on media supplemented with several concentrations of 2iP alone induced callus formation and *C.canephora* produced callus on media supplemented with 2iP and 2,4 D [36, 37].

In this study, friable embryonic callus and compact callus were observed on Geisha and N39-6 explants cultured on MS media with BAP (4 mg/L) and 2,4 D (1 mg/L) (Fig. 3) respectively. The compact callus displayed a whitish heavy mass whereas the friable callus was a fragile, soft-yellowish mass as explained before in a histological study for *Dioscorea nipponica* Makino [38]. In the case of coffee, similar results have been demonstrated from leaf explants of *Coffea arabica L. var. Colombia* [12]. Geisha explants produced many friable callus whereas compact callus were dominated by N39-6 explants. This might explain the possibility of the lower average weight of embryonic callus for Geisha compared to N39-6. Besides the callus features, the low ratio of 2,4 D to BAP has been proven to promote embryonic callus in leaf explants of *Coffea arabica var Ababuna* (hybrid) and *Coffea arabica L. var. Colombia* [25, 26]. Their reports aligned with the current embryonic callus formation observed on Geisha and N39-6 leaf explants established on MS with 2,4 D (1 mg/L) and BAP (4 mg/L) media (Fig 3A and 3B). Our findings showed that auxin and cytokinin interaction plays a major role in coffee callogenesis.

This current study demonstrated that friable callus and compact callus established on MS-BAP media alone produced somatic embryos on Geisha and N39-6 and induced the different stages of embryo maturation like globular, torpedo, and heart-shaped (Fig 4A; 4B and 5A; 5B). These findings aligned with a current report from Avila Victor CM et al.,2023 elaborated that embryo maturation occurred in both friable and compact callus of *Coffea arabica L. var. Colombia* [39]. In our observation, Geisha produced many somatic embryos from friable calli whereas N39-6 somatic embryos arose from compact calli. Moreover, different studies elaborated on the role of cytokinin in somatic embryo maturation specifically

on torpedo and heart-shaped embryos [40]. Particularly in coffee, BAP alone has been reported to induce somatic embryo maturation of Coffea liberica [41]. Conversely, de Morais Oliveira et al., 2023 reported that auxin (2,4-D) promotes abnormal embryo development resulting in immature coffee plantlets germination [42]. This study achieved relative results using MS media supplemented with 1.125 mg/L of BAP alone proving the role of cytokinin in somatic embryo development.

Half MS media supplemented with BAP and biotin in germination media (T4) promoted the rooting stage of Geisha and N39-6 plantlets (Fig. 4C and 5C). Cytokinin and auxin play an antagonistic interaction role in (SAM) shoot and RAM (root) development [43]. Previous reports showed the potent outcomes of cytokinin for shoots and root elongation on coffee plantlets, unlike auxin which was reported to influence several malfunctions in shoot development [44-47]. According to a study by Ruiz et al., 2019, they used biotin to growth media, and that formulation enhanced root formation in Arabidopsis thaliana [48]. This study enlightened the importance of cytokinin and vitamins in the coffee somatic embryo germination. The current study showed that Geisha and N39-6 plantlets sustained the gradual adaptation in *ex vivo* conditions and produced complete coffee plantlets (Fig. 4D and 4E) and (Fig. 5D and 5E). It was explained before that the success of in vitro micropropagation is determined by the ability of the plantlets to adapt external environment [48, 49]. This protocol succeeded in regenerating matured coffee seedlings from callus-induced leaf explants. However, further study is mandatory to investigate possible approaches that may shorten the acclimatization duration of these plantlets.

Conclusion

The current study demonstrates the potency of indirect somatic embryogenesis protocol using auxin-cytokinin interplay from Geisha and N39-6 hybrids leaf explants. Although the success of indirect somatic embryogenesis depends on a particular coffee genotype, this protocol shows the potential regenerative ability of the Geisha and N39-6 variety. However, ongoing standardization of these plant growth regulators and media formulas is mandatory to influence mature embryo transformation to plantlets for mass production of these varieties.

Acknowledgement

Special thanks to Mrs Glory Kweka for her continuous assistance in all the experiments performed and Ms. Neyonkulu Kahisha for proof reading this manuscript.

Funding

This work was supported by Tanzania Coffee Research Institute (TaCRI).

Authors Contribution

Ms. Fatuma Jumapili Ramadhani and Dr. Damian Mtenga conceptualized, designed and wrote the manuscript. Ms. Fatuma Jumapili Ramadhani and Ms. Rehema Erasto Mwaipopo conducted the experiment, interpreted and analyzed data. Dr. Damian Mtenga and Dr. Deusdedit Kilambo managed the project and supervised experiment progress. Mr. Nuhu Mbwebwe Aman and Dr. Suzanna Mbwambo revised and edited the manuscript. All authors read and approved the final manuscript.

Competing Interest

The authors declare that they have no competing interest

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