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Review Article

# Hybrid Coffee (*Coffea arabica* L) Plantlet Production via Indirect Somatic Embryogenesis in Ethiopia: Current Statue and Future Direction

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## Abstract

Traditional hybrid coffee propagation methods using seed or vegetative cuttings has tremendous limitations. Seed propagation is associated with hand pollination which is time consuming and need large number of skilled labor. On the other hand, vegetative cuttings ensure uniformity, but; cuttings generate relatively low multiplication rates as they can only be obtained from orthotropic branches. Multiplication by tissue culture techniques could provide a best alternative to these traditional methods of coffee propagation as it allowed the production of relatively uniform plantlets on a large scale in a shorter period in any climatic condition. Among tissue culture techniques, indirect somatic embryogenesis of hybrid coffee plays substantial role in rapid industrial scale multiplication of high valued varities. Research on somatic embryogenesis of *C. arabica* hybrid has been conducted since the late 70s with the objectives to substitute the conventional vegetative propagation of selected varieties. In Ethiopia, in vitro propagation of hybrid coffee is recent phenomenon and research on indirect somatic embryogenesis is already well underway by different research groups. Here, we discussed the current status of coffee research on somatic embryogenesis in general and specifically, we provide recommendations for future research for the establishment of mass propagation protocol for F1 hybrids varities in Ethiopia that utilized wild endogenous lines.

## Keywords

Indirect Somatic Embryogenesis, Hybrid Coffee, Somatic Embryo, Mass Propagation, *C. arabica* L

## 1. Introduction

Ethiopian economy mainly relies on export of agricultural out puts. Among export agricultural commodities, coffee plays a major role in country's economy. Ethiopia is Africa's leading coffee producer and the fifth largest in the world with 384,000 metric tons and the third largest producer of arabica coffee [1]. Coffee is one of the major income generating commodity for Ethiopian economy as it contributes 25-35% of the total export earnings [2]. Beyond this, using DNA-based genetic markers, it was also confirmed that Ethiopia is the natural habitat and primary center of origin of *Coffea arabica* [3, 4]. This immense genetic recourse provides huge opportunity for coffee improvement through pure line selection and hybridization for yield, disease resistance, climate change resilience and quality as well as other economically important traits.

Despite the existence of genetic variation for improvement,

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the annual average yield of green coffee is considered as very low ~ 6 qt/ha [5]. The presence of old and poorly managed farm trees, lack of improved cultivars, occurrence of disease such as coffee berry disease, leaf rust and coffee wilt are among the major factors for this low productivity [6, 7]. Furthermore, climate change also contributes enormously for the reduction of yield [8].

In order to address this production constraints, Jimma Agricultural Research Center (JARC) developed three hybrid coffee cultivars Aba buna, MCH2 and Gawe with average yield of 23.8, 24, and 26 qt/ha, respectively [9]. In addition, higher hybrid performance for yield (18%-60%) among the cross of elite breeding materials in Ethiopia [10]. So, the demand for hybrid seed and seedling from coffee farmers as well as from coffee plantation owners is high. However, those high yielding coffee varieties were not distributed to coffee growers, due to lack of efficient seedling production techniques. Currently, coffee research institutes in Ethiopia producing hybrid seed through conventional approach that is inefficient [9]. Therefore, an economically feasible, clonal propagation method is needed to satisfy the growing demand of hybrid seed in order to make effective use of observed yield advantage.

Conventionally, hybrid coffee is propagated from seed or by vegetative cuttings. Seed propagation is associated with hand pollination to get F1 seed. This method is time consuming and need large number of skilled labor. Consequently, using F2 leads to lack of uniformity as a result of segregation [11]. Propagation of coffee by vegetative cuttings guarantees uniformity. Cuttings generate relatively low multiplication rates as they can only be obtained from orthotropic branches. Multiplication by tissue culture techniques could provide a best alternative to these traditional methods of coffee propagation [11]. Tissue culture methods permit the production of relatively uniform plants on a massive scale in a shorter period, and with a narrower genetic base than those under the conventional methods [12].

Several studies have been carried out with the aim of micropropagation of superior coffee genotypes by using apical or axillary meristem and nodal cultures [13]. All these techniques are extremely important in the multiplication of genotypes although the average rate of multiplication is not very high. For instance, [14] in which 2.8 shoots per node on average was obtained using 44.4 μM BA on *C. arabica*. Likewise, Ribeiro and Carneiro [15] also obtained 6.8, 2.5 and 2.7 shoots per node on average from three *C. arabica* cultivars, Caturra, Geisha and Catimor, respectively. However, application of somatic embryogenesis (SE) is a highly useful method for the large-scale propagation of economically important plants such as coffee [16]. Embryogenic callus formation offers great potential for promoting culture efficiency whereby somatic cells are induced to differentiate embryogenic cells and form somatic embryos that develop into new plants [17].

Application of bioreactor technologies is one of the major factor that can improve tissue culture yield at low cost. Two major bioreactor types, RITA and MATIS, with temporary immersion system were released for coffee *invitro* propagation with different capabilities in 2007 and 2013, respectively. The RITA has some advantage such as its simplicity and reliable operation, the compact space for apparatus accommodation, and the support of sufficient relative humidity level with full separation of the propagules and liquid medium [18]. The main disadvantages of this systems are the inability for nutrient medium renewal and the lack of options for forced ventilation and $CO_2$ enrichment [18]. On the other hand, MATIS bioreactor designed especially to favor embryo dispersion and light transmittance to SE, improved the embryo-to-plantlet conversion rate in *C.arabica* [19]. Recently, Plantform$^{TM}$ bioreactor come a long with several advantage: It is easy to handel, transparent, autoclavabel. As compared to RITA and MATIS, Plantform bioreactor capable to control gas exchange through the use of air pumps and timer [20]. This forced ventelation leads to the complete renewal of the culture's atmosphere which prevent the accumulation of CO2 and Ethylene. Beyond this, unlike RITA and MATIS, Plantform bioreactor apparatus can be stacked one on the other to save space [20]. Having all these benefits, nobody reported application of Plantform bioreactor for *C.arabica invitro* mass multiplication using nodal culture as well as somatic embryogenesis.

The ability to initiate embryogenic cultures is controlled by the internal and external factors of the explants, including the genotype, the developmental stage, and the different tissue of the explants, as well as medium composition [21]. Alternatively, the methodologies to induce somatic embryogenesis and the further development of embryos are genotype-dependent, which leads to an almost practically development of protocols for each cultivar or clone [22]. Wide range of protocols available in the literature confirms that coffee is not a recalcitrant species for somatic embryogenesis. However, significant range of variability to successful regeneration has been reported. Large number of different protocols exist that corresponds to large number of cultivars, show a different outcome to the same protocol. A better understanding of the processes as a whole will clarify the most important and specific bottlenecks and leads to effective solutions. In this regard, molecular markers are key to improve the whole process of SE and the step toward full commercialization [22]. The strength of molecular markers is the prediction of the embryogenic capacity of calli before they have differentiated into embryos and to use this information to modify the current protocols.

To apply different novel *in vitro* technologies, combining with the latest omics techniques (genomics, transcriptomics, proteomics, metabolomics, and phenomics) help to optimize the production process of somatic embryogenesis and the subsequent conversion into rooted plantlets [22]. This also help in identifying effective molecular marker. In this regard, recently, identified 1052 non-redundant proteins, being 5 only

annotated to embryogenic capacity. Many proteins are without any annotation and so are still uncharacterized, showing the poor molecular knowledge about somatic embryogenesis in coffee [23].

## 2. Conventional Techniques for Coffee Propagation

Coffee varieties associated to Arabica and Robusta are traditionally propagated by seeds [24]. The main limitations for hybrid coffee plantlets production is associated with the segregation of F1 hybrid cultivars, the short span of seed viability and the long juvenile (4 years). Hybrid Arabica varieties are sold in the form of seed after long pedigree selection process of at least 20 years [25].

Coffee clonal vegetative propagation can be done by grafting or cuttings and axillary or adventious shoot propagation. These techniques have many limitations such as: (1) The risk of disease dissemination [25]. (2) The number of orthotropic cuttings obtained from shoot or bud propagation is limited (4 or 5 per plant). (3) Clonal garden establishment for shoot production leads to extra cost [10, 16]. (4) very poor rooting frequency of vegetative cuttings, particularly in Arabica [10]. Therefore, manual hybrid seed production and cuttings are costly and tedious on Arabica and calls for alternative techniques.

## 3. Somatic Embryogenesis and Its Application in *Coffea arabica*

### 3.1. Somatic Embryogenesis

Somatic embryogenesis (SE) is a biological process in which, in presence of the right stimulus such as plant hormones and/or stress, embryogenic cells are generated from somatic cells, and through a series of biochemical and morphological changes, perfectly organized embryos are attained [26]. In other words, it is a process of differentiation of cells into a plant bypassing the fusion of gametes. It is also the powerful tool in biotechnology for propagation of species with a long reproductive cycle or low seed set and production of genetically modified plants with improved traits [27]. Embryogenic cells has two important attributes; they are able to proliferate, which makes SE suitable for mass production of elite cultivars and plantlets can be regenerated from one single cell [28]. The occurrence of SE is achieved only if the cells are competent enough and receive an appropriate inducing stimulus [27].

Plant regeneration through SE includes five common steps based on review on [29]: (1) induction of embryogenic callus on medium mainly supplemented with 2,4-D, (2) the multiplication of embryogenic cells on solidified or liquid medium (3) somatic embryo development in medium lacking 2,4-D (4) embryo maturation (5) the development of plants.

Somatic embryos can be obtained in two different ways, directly or indirectly. Direct somatic embryogenesis occur when the embryos are formed without intermediate callus formation (proliferation of cells) directly on the explant [30]. Whereas, indirect somatic embryogenesis occur first an embryogenic callus is formed and then embryos arise from this callus [31, 32]. In the indirect way, two distinct phases are involved, called induction and expression. The induction stage is marked by changes in the metabolism and gene expression, leading to the differentiation into embryos in the expression phase [31, 33, 34].

Direct SE require short period to regenerate embryos than indirect methods and this advantage could be exploited as an advantage for the rapid release of the coffee plantlets (e.g. 70 days in coffee). However, quantitatively indirect SE is more advantageous than direct SE. In indirect SE a friable embryogenic callus is formed either as calli or cell suspensions and up on transfer to 2,4-D free media leads to embryo regeneration [17].

### 3.2. Applications of Somatic Embryogenesis in Coffee

SE provides the best alternative to other vegetative propagation systems as it provides industrial scale production of embryos. Nowadays, coffee somatic embryogenesis is considered as a model system to study cellular mechanisms for perennial species [34]. Different protocols and methods in SE developed for the past 50 or more years for genotypes of both commercial coffee species, is well represented in literature [35-42]. For *Coffea arabica*, the first stape is inducing primary callus using auxin or 2,4-D containing medium for using $1cm^2$ leaf as explant (2.26 μM of 2, 4-D, 8.69 μM of 2iP and 4.92 μM of IBA) then after 30 day transferred to embryogenic callus induction medium with 2, 4-D and BA (4.52 μM and 17.75 μM, respectively). Finally, the obtained embryogenic callus either multiplied in cell suspension culture or transferred to regeneration medium that contains only with cytokinin (4.4 or 17.6 μM BA) [43].

In general, embryogenic calli are yellow and friable and their cells are small, isodiametric, arranged in clusters, with a dense cytoplasm, a nucleus with salient nucleoli and rich in small amyloplasts [17, 22, 43]. Whereas, non-embryogenic calli are spongy and translucent showing cells that are more elongated, with the vacuole occupying a big volume of the cytoplasm, higher number of vesicles and absence of cytoplasmic organelles [22]. Moreover, the developmental stages in SE include the globular, heart-shaped, torpedo, and cotyledon of embryo development [27]. Recently, industrial scale of multiplication of hybrid coffee plantlets achieved using temporary immersion bioreactors that allowed mass production of fully germinated cotyledon stage embryo in liquid medium and subsequent acclimatization [44]. Therefore, indirect somatic embryogenesis method was the ultimate preference for scaling-up and development of industrial

propagation procedures using bioreactors technology.

### 3.3. Coffee *in vitro* Mass Propagation in Bioreactor

The bioreactor is specialized technological equipment, designed for mass *in vitro* culture by regulating various nutritional and/or physical factors [20, 22]. Large scale cultivation of differentiated (embryos, shoots, seedlings, transformed or adventitious roots) and dedifferentiated (suspended cells) plant cultures could be realized by growing them *in vitro* in liquid media, under controlled environmental conditions in bioreactor systems. The major objective of using this technology is to achieve economically feasible production of maximal amounts of plant biomass, ready for direct application [20].

Extensive research has been conducted for development of new bioreactors and techniques for mass multiplication of somatic embryos and also to improve embryo-plantlet conversion [19, 44-48]. In vitro mass multiplication of hybrid *C. arabica* somatic embryo has been conducted using disposable bags, erlenmeyer flask and bioreactors in liquid medium [17, 19, 45]. Bioreactors of different capabilities have been introduced for coffee invitro mass propagation including stirred bioreactors [45, 46], temporary immersion bioreactors like 1 L RITA R "Récipient àImmersion Temporaire Automatique" [17], and 10-L horizontal disposable plastic bags [45]. Using those bioreactors, preparation of cell suspension from embryogenic callus is a key step for scales up the number of embryos for large scale production [45, 46].

However, embryo-plantlet conversion and genotype difference remain another key factors for successful application of bioreactor for mass production of embryo and reproducibility of protocols [19]. With regard to genotype difference, response to somatic embryogenesis was observed among the F1 arabica coffee hybrids. In case of genotypes with better embryogenic response, up to 9000 plantlets were harvested per RITA bioreactor, but in the case of a low-embryogenic hybrid, only 750 to 1000 plantlets were obtained per vessel [17]. This implies, the need for protocol optimization for each coffee cultivar is mandatory. Recently, the use of a new MATIS® bioreactor with surface area of 355 $cm^2$ used to improve embryo plantlet conversion rate by nearly 91%. This improvement is largely possible owing to the design that specially to favor embryo dispersion and light transmittance to somatic embryos [19].

Application of bioreactor technologies is one of the major factor that can improve tissue culture yield at low cost. Two major bioreactor types, RITA and MATIS, with temporary immersion system were released for coffee *invitro* propagation with different capabilities in 2007 and 2013, respectively. The RITA has some advantage such as its simplicity and reliable operation, the compact space for apparatus accommodation, and the support of sufficient relative humidity level with full separation of the propagules and liquid medium. The main disadvantages of this systems are the inability for nutrient medium renewal and the lack of options for forced ventilation and $CO_2$ enrichment [18]. On the other hand, MATIS bioreactor designed especially to favor embryo dispersion and light transmittance to SE, and improved the embryo-to-plantlet conversion rate in *C.arabica* [19]. Recently, Plantform™ bioreactor come a long with several advantage: It is easy to handel, transparent, autoclavabel. As compared to RITA and MATIS, Plantform bioreactor capable to control gas exchange through the use of air pumps and timer [20]. This forced ventelation leads to the complete renewal of the culture's atmosphere which prevent the accumulation of CO2 and Ethylene. Beyond this, unlike RITA and MATIS, Plantform bioreactor apparatus can be stacked one on the other to save space [20].

Different researchers published the successful application of Plantform bioreactor. The first evidence of the effectiveness of liquid culture in temporary immersion system by Plantform bioreactor reported in promoting shoot proliferation of *pedunculate oak* [49]. Likewise, [50] for the first time reported application of Plantform bioreactor on olive shoot multiplication. They found that, the Plantform™ bioreactor improves the *in vitro* culture of *Olea europaea* L. cv Canino, by inducing higher proliferation, increasing shoot length and enhancing the vigour of shoots in comparison to the semi-solid culture. The use of the Plantform bioreactor also successfully solved the problems related to scale-up of date palm micropropagation, such as asynchrony of somatic embryos, limited maturation of somatic embryos, and highly variable germination frequencies of embryos [51]. Having all these benefits, nobody reported application of Plantform bioreactor for *C.arabica invitro* mass multiplication.

## 4. RNA-Seq: Transcriptome Profile of Somatic Embryogenesis

Recent progress in systems such as next-generation sequencing (NGS) and bioinformatics techniques have revolutionized 'omics' research in plant science [52]. The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues [52, 53]. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns and other post transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions [52].

RNA-Seq is a recently developed approach to genome-wide transcriptome profiling that uses deep-sequencing technologies. Extensive research have been conducted using

RNA-Seq to transcriptomes of different plants to compare and identify differentially expressed genes. For instance, [54] identified differentially expressed genes during somatic embryogenesis in Fragaria ×ananassa. Likewise, [55] identified differentially expressed patterns from cotton with different somatic embryogenesis capabilities. To understand the molecular process of embryogenic callus formation in wheat, [56] applied RNA-Seq and identified differentially expressed genes.

RNA-Seq technology also implemented by different researchers in *C.arabica*. For instance, [57] applied RNA-Seq and DNA-Seq on two distinct *C.arabica* accessions and used *C. canephora* as reference genome. They found that, early evolution of *C.arabica* mainly involved homologues cross over exchange. [58] obtained a transcriptome profile of coffee rust interaction and identified putative unregulated resistant genes. Transcriptome of leaves, flower and fruits perisperm of *C.arabica* was also analyzed and differentially expressed genes related to raffinose biosynthesis identified [59]. Do santos [60] investigated transcriptome of *C. arabica* root under Nitrogen starvation for the first time. They obtained 34,654 assembled contigs by RNA-Seq and validated 12 genes by RT-qPCR. However, nobody conducted RNA-Seq analysis on *C. arabica* calluses to identify genes related to high embryogenic capacity trait.

Recently, 'omics' technologies also applied to identify differentially expressed genes by comparing embryogenic and non-embryogenic callus in Arabica coffee. [61] compared embryogenic and non-embryogenic callus cluster using RNA differential display analysis. In this work, the comparison was done only through counting or comparing specific bands on gel. In an attempt to understand SE in coffee plant, [62] analyzed differential protein profiles of three stages of SE, including globular, torpedo and cotyelodon somatic embryos of Coffea arabica cv. catuai Vermelho. As a result, they found proteins (11S storage globulin proteins) that could be used as molecular marker. Similarly, [23] identified 1052 non-redundant proteins of which 5 of them were annotated for embryogenic capacity. The major drawback of proteomic data is that low abundant proteins not detected [63]. Therefore, RNA-seq the most preferred method to study differentially expressed genes for the crop like *C. arabica* as its full reference genome is available recently and there is no work related to application of RNA-SEQ technology on somatic embryogenesis of wild Ethiopian coffee.

## 5. Future Research Direction

Better understanding of embryogenic capacity of commercially important perennial crops like C. arabica is extremely important in order to enhance coffee breeding efficiency and in vitro mass multiplication of hybrids. Therefore, this review pinpoints four main research gaps that should be tackled in the future research activities.

First, it is well known that, primary center of diversity for *Coffea arabica* L is found in the south-western highlands of Ethiopia. The total number of *Coffea arabica* accessions conserved at Jimma Agricultural Research Center was about 5317. The current research effort mainly focused on improvement of limited number of traits such as high yield, disease resistance and quality through pure line and hybrid breeding [9]. Despite the presence of such immense genetic diversity, none of those genetic resources evaluated for their embryogenic capacity. This parameter is extremely important for *in vitro* mass propagation of elite hybrid coffee varieties through direct or indirect somatic embryogenesis at reasonably low cost.

Second, genes responsible for high embryogenic capacity, which could be otherwise used as marker, not yet identified. In response to stimuli, wide range of responses in plant cell observed at molecular, biochemical and physiological level. At molecular level the response to embryogenic callus formation results in differential expression of several metabolic pathways [53]. For this reason, exploring the transcriptional activity (with coding and non-coding) and gene profiling for global picture of cell function and difference in gene expression of high and low embryogenic genotype enables the identification of high embryogenic related genes that could be used as marker for selection of high embryogenic traits among breeding lines. Therefore, genome wide RNA-Seq technology should be employed to compare coffee genotypes with contrasting embryogenic capacity.

Thirdly, problems related to protocol optimization for released commercial hybrid coffee varieties will be addressed. Currently, there is no workable protocol for *in vitro* mass multiplication of Ethiopian hybrid coffee varieties at large scale. Using Ethiopian hybrid, Aba buna, the highest number of embryos repored that germinated per explant was (14.0 ± 1.7) on MS medium supplemented with 2.0 mg /l BAP in combination with 0.5 mg /l gibberellic acid (GA3) [41]. On the other hand, [28] reported 70,000 embryos per gram of Embryogenic callus on *C.arabica* cv 'Catimor'. This is extreme difference calls for further protocol optimization. Optimizing the somatic embryogenesis process at each stage is mandatory for both direct and indirect somatic embryogenesis. Among the different developmental stages, embryo-plantlet conversion was identified as the main bottleneck worldwide [45]. Therefore, to improve embryo-plantlet conversion, bioreactor technologies should be employed and investigated.

Finally, significant advances in coffee somatic embryogenesis have enabled the production of large numbers of aseptically grown coffee plants from orthotropic leaf cultures [22]. Despite the high multiplication rate, its application is limited by the indeterminate nature of embryogenic cultures, the long period of time from culture initiation to embryo production (6–12 month.), and the relatively high cost per plant produced [64]. Having these limitations in mind, micropropagation procedure should be developed on coffee based on somatic embryo-derived plantlets of apical and nodal stem explants of hybrids. Such system may help for massive production of ax-

illary shoots by increasing the efficiency and reducing the costs. [65] performed such experiment on Cacao and obtained encouraging result that would potentially increase the multiplication rate, reduce the propagation period, decrease the dependency on new culture initiation, and avoid multiple somatic embryogenesis. Such a system could increase the efficiency of the system and reduce the associated costs.

## Abbreviations

| | |
|---|---|
| 2,4-D | Dichlorophenoxyacetic Acid |
| 2-iP | Dimethyl-allylamino Purine |
| IBA | Indole Butyric Acid |
| BAP | 6-benzylaminopurine |
| JARC | Jimma Agricultural Research Center |
| NGS | Next Generation Sequencing |

## Author Contributions

Elyas Gebremariam is the sole author. The author read and approved the final manuscript.

## Conflicts of Interest

The author declares no conflicts of interest.

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