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In vitro multiplication of *Coffea arabica* L. from leaf explants through indirect somatic embryogenesis

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Abstract
Leaf explants of approximately one year old C. arabica L. plant samples from India were surface sterilized and cultured on MS (Murashige and Skoog) medium containing 30g/l sucrose, 4g/l agar and 10mg/l ascorbic acid. Out of the different MS media supplemented with different combination of auxins and cytokinins tested in a separate experiment for callus induction from the leaf explants, only six auxin/cytokinin combinations induced callus formation. Callus induction occurred in the range of three to four weeks after the inoculation of the explants. Each callus initiated on specific culture medium was sub cultured on identical but fresh medium at an interval of four weeks after callus initiation. After two months of incubation in the callus induction medium, the calli were transferred on to half strength MS medium of their respective callus induction medium for the initiation of somatic embryos and plantlets. After four weeks of incubation on these half strength MS media somatic embryos developed on the surface of the callus as green color. In the subsequent additional sub-culturing/month for four months on the same half strength MS media, the developed somatic embryos matured and germinated into plantlets (shoots). The 10 cm shoots were rooted on and acclimatized.The results were discussed from identification of the best growth hormone combinations and their respective concentrations suitable for Coffea Arabica L. in vitro multiplication from leaf explants through indirect somatic embryogenesis.

Keywords: auxins c. arabica, callus, cytokinins, explants, in vitro multiplication, somatic embryos

Introduction
Coffee provides one of the most widely consumed beverages in the world. It is an important agricultural export commodity in more than 50 developing countries of Africa, Asia and Latin America (Dublin *et al.*, 1991) [5]. Botanically, coffee belongs to the family Rubiacae and is classified taxonomically under the genus *Coffea* which includes at least 64 species grouped into four sections (Carvalho and Monaco, 1969).

Coffea Arabica L. is a species that originates from the Ethiopian highlands (Cros, 1998). All the species of this genus have 2n = 2x = 22 chromosomes, apart from the notable *C. arabica* 2n =.4x =44. Self-fertilization of the species is not absolute, with selfing being estimated at 90% under plantation conditions (Carvalho, 1988) [3]. The commercial coffee productions relies on two species of coffee, *Coffea arabica* L. and *Coffee canephora* Pierre ex. Froehn, with *C. arabica* being considered as superior quality coffee, and contributing to over 70% of the world's coffee production (Orozoco-Castillo *et al.*, 1994) [14].

Coffea arabica varieties (allotetraploid self-fertilizing) are sold in seed form as more or less fixed “pure lines” after a relatively lengthy pedigree selection process, taking at least 20 years. Seed propagation is associated with inherent uncontrolled genetic variation in the heterozygous cultivars, slow rates of seed multiplication, short span of seed viability (Monaco *et al.*, 1995) [11].

Recently, in vitro culture has played an important role in agriculture and plant science. This method allows the production of large number of genetically identical plants which can be produced from a single mother stock (Shibli, *et al.*, 1997) [20].

Plant production via tissue culture is advantageous over traditional propagation methods because it leads to the production of disease and virus free plants (Shibli, 1995) [19]. It also allows the production of a high number of plants in a short period of time and in a very limited propagation space (Shibli, 1995) [9]. In addition, rapid multiplication rate of plants that are difficult to propagate conventionally can be easily achieved through in vitro culture (Carneiro and Ribeiro, 1989) [2]. Various approaches have been considered for in vitro multiplication of coffee (C. Arabica) from apical meristem and axillary bud culture, induction and development of adventitious buds (Carneiro and Ribeiro, 1989) [2] and somatic embryogenesis (Staristsky, 1970). Various tissues such as orthotropic and plagiotropic shoots (Staritsky, 1970; Nassuth *et al.*, 1980; Raghuramulu *et al.*, 1987) [23, 13, 17], leaf tissue (Söndahl and Sharp, 1977; Quiroz-Figueroa *et al.* 2002a, 2002b) [21, 16], ovule integument (Lanaud, 1981) [7], and somatic tissue from anthers (Ascanio and Arcía, 1987) [1], and perisperm (Sreenath *et al.*, 1995) [22] have been used to induce callus or somatic embryos or combinations of both in coffee. Leaves are so far the most widely used source of explants because of their year-round accessibility, and they can be readily obtained at different developmental stages (Santana *et al.*, 2007) [18]. Therefore, the aims of this investigation were to identify the best growth hormone combinations and their respective concentrations suitable for *Coffea Arabica* L. in vitro multiplication from leaf explants through indirect somatic embryogenesis.

Materials and Methods
Tissue culture media
The tissue culture media used in this study were based on Murashige and Skoog (1962) [12]. All the essential elements

were grouped in to four categories supplemented with plant growth hormones, carbon sources and solidifying agent (table1).

Preparation of MS media stock solutions

Because of difficulties and tidiness of weighing and mixing all the ingredients of MS medium at the time of media preparation, it is advisory to prepare concentrated solutions of the different categories of ingredients and store them in a refrigerator at 5º C for later use (table2-3). Each MS stock solution needs to be renewed after each month.

Table 1: Components and concentration of MS media stock "A" solution

Categories	Components	200x Conc. in mg/l of distilled water	1x conc. in mg/l of distilled water
Macronutrients (Stock "A")	NH_4NO_3	33000	1650
	KNO_3	38000	1900
	$CaCl_2.2H_2O$	8800	440
	$MgSO_4.7H_2O$	7400	370
	KH_2PO_4	3400	170

Table2: Micronutrients (Stock B)

Categories	Components	200x Conc. in mg/l of distilled water	1x conc. in mg/l of distilled water
Micronutrients (Stock B)	KI	166	0.83
	H_3BO_3	1240	6.2
	$MnSO_4.4H_2O$	4460	22.3
	$ZnSO4.7H_2O$	1720	8.6
	$Na2MoO_4.2H_2O$	50	0.25
	$CuSO_4.5H_2O$	5	0.025
	$CoCl_2.6H_2O$	5	0.025
	Na_2EDTA	7460	37.3
	$FeSO_4.7H_2O$	5560	27.8

Table3: Organic supplements (Vitamins and Glycine) (Stock C)

Categories	Components	200x Conc. in mg/l of distilled water	1x conc. in mg/l of distilled water
Organic supplements (Vitamins and Glycine) (Stock C)	Myo-inositol	20000	100
	Nicotinic Acid	100	0.5
	Pyridoxine-HCl	100	0.5
	Thiamine-HCl	100	0.5
	Glycine	400	2

Preparation of Auxins (IAA, IBA, NAA and 2,4D) stock solutions

20 mg of each of Indole acetic acid (IAA), Indole butyric (IBA) and Naphthalene acetic acid (NAA) were dissolved separately in 2-5ml of diluted NaOH and diluted to a final volume of 100 ml of distilled water and stored in a refrigerator in separate bottles for later use. 20 mg of 2, 4-dichlorophenoxy acetic acid (2,4D) was dissolved in 2-5 ml of ethanol and then diluted in a final volume of 100 ml distilled water and stored in a refrigerator in a separate bottle for later use.

Preparation of Cytokinins (BAP and Kinetin) stock solutions

20 mg of 6- Benzyl amino purine (BAP) and 20mg of kinetin (kin) were dissolved separately in few drops of hydrochloric acid (HCL) and ethanol, respectively. Each was then diluted to a final volume 100ml in distilled water and stored in a refrigerator in separate bottles for later use.

Preparation of working solution of MS culture media

To prepare a liter of working solution of culture medium, 30 gram of sucrose, 50ml of stock "A" (macronutrients) and 5 ml of each of the other two stock solutions (stocks B, and C) are dissolved in double distilled water and then the required amount of growth hormones are added before the final volume is adjusted to a liter with double distilled water followed by pH adjustment to 5.8 using diluted solutions of sodium hydroxide (NaOH) and hydrochloric acid (HCL). Then the mix is boiled on hot plate before adding 6 mg/l Agar or 4 mg/l cleriGel by stirring thoroughly until they dissolve completely, before dispensing approximately 25ml to each culture vessel and autoclaved at 121 º C and 15lb pressure for 20 minutes.

Experimental plant materials

Approximately a year old seedlings of *C. arabica* were obtained from Mr. V. G. Broom's privately owned indigenous and exotic spicy and medicinal plants artificial park situated in Marunji village 10km Northwest of Pune City, Maharashtra, India.

Establishment of *C. arabica* seedlings in the greenhouse

The *C. arabica* seedlings were established in the greenhouse of the Department of Botany Dr. Babasaheb Ambedkar Marathwada University Aurangabad, Maharashtra, India. Leaf explants from the seedlings were used for the induction of calli and somatic embryos.

Surface sterilization of leaf explants

Surface sterilization of coffee leaf explants from green house was accomplished by careful selection of undamaged and healthy leaves and washing them in distilled water containing few drops of tween 20 for 10 min. and then

immersing them in 0.3% mercuric chloride ($HgCl_2$) for 5 min. followed by rinsing with autoclaved distilled water three times (5 min each).

Induction of callus from leaf explants of *C. arabica* L.

Surface sterilized leaf explants were cultured in MS medium (Murashige and Skoog, 1962) [12] containing 30g/l sucrose and 4g/l agar. Different concentrations of auxins (0.2, 0.4, 0.6, 0.8 and 1 mg/l of auxins IAA, NAA, IBA and 2, 4D) with varying concentrations of cytokinins (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5mg/l of cytokinins BAP or kinetin) were tested in a separate experiment for each auxin and cytokinin combinations to induce callus from *C. arabica* leaf explants considered in the present investigation. Each treatment consists of 10 replicates in a completely randomized design. The pH of the medium was adjusted at 5.8 and autoclaved at 121º C for 20 minutes. Approximately 25 ml of medium was dispensed in glass vessel with 10 replicates each. After inoculation of surface sterilized leaf explants, the culture were maintained in growth room at 26 º C ± 2 º C. for callus initiation. The cultures were observed for growth or contamination every day after inoculation and incubation.

From the preliminary callus induction experiments, the nutrient media consisting of specific auxin and cytokinin combinations which initiated callus formation were considered. The growth of the callus was judged visually and in terms of increase in biomass on the basis of fresh weight (FW) and dry weight (DW). After three rounds of sub-culturing on callus induction media, sample callus from each specific medium was removed from debris of explants if any, and its fresh weight was determined. This was followed by air drying of each sample callus for 48 hrs under fluorescent light before it was reweighed for the determination of its dry weight. The moisture content of each sample callus from specific medium was determined as percentage from its fresh weight and dry weight. Moisture % = FW-DW/FWx100.

Induction of somatic embryos and plantlets from induced calli of leaf explants

The induced calli on MS media supplemented with specific auxins and cytokinins combinations (1 mg/l each of 2,4D and BAP; 1.5 mg/l BAP and 0.5 mg/l 2,4D; 1.5 mg/l BAP and 0.2 mg/l 2,4D; 3 mg/l BAP and 0.2 mg/l 2,4D; 4.5 mg/l kin and 0.5 mg/l NAA; and 4.5 mg/l kinetin 0.2 mg/l NAA) were sub-cultured on similar but fresh medium every month. After two months of incubation in the callus induction medium, the calli were transferred on to half strength MS medium of their respective callus induction medium for the initiation of somatic embryos and plantlets.

Rooting of micro-shoots

Rooting was carried out by sub-culturing 10 mm long micro-shoots in culture vessels containing 25 ml of solid half-strength MS media containing 15gm/l sucrose. The media were supplemented with IAA, IBA, NAA or 2,4D separately at 0.0, 1.0, 2.0 or 3.0 mg/l. Experiments were arranged in a Completely Randomized Design (CRD) with 5 replicates. The cultured micro-shoots were maintained under 26±2 °C with 16 hours light and 8 hours dark. Data were collected on number of roots, root length and shoot height after 30 days.

Acclimatization

Acclimatization was carried out by opening culture vessels for 3 days before transferring plantlets outside of the growth chamber. *In vitro* rooted plantlets were extracted from culture vessels and the agar was removed by washing with warm sterile water. The plantlets were transferred to plastic bags containing sand and peat mixture with equal proportion. The plastic bags containing plantlets were covered with transparent plastics with holes to minimize transpiration and facilitate sufficient air circulation, respectively. For the first three to four weeks the plants were placed under shade with low light intensity and high humidity at a temperature of 26±2ºC, and were irrigated with $1/4^{th}$ strength MS media at an interval of two days. Then after, the seedlings were transferred to natural field conditions and percentage of their survival was recorded in both phases of acclimatization experiments.

Statistical Analysis

Each experiment was setup as a completely randomized design. The collected data was statistically using one-way ANOVA. Statistical Analysis System (SAS) software version 9.1.3 and SPSS statistical software version 16 were used. Means were separated according to the least significant difference (LSD) test at 0.05 level of probability.

Results and Discussion

The present study was designed to optimize tissue culture technique for *C. Arabica* L. Different parts of the plant were tested for their potential use as explants in *C. arabica* in vitro multiplication. Different parts have differential responses to different concentrations of auxins and cytokinin combinations.

Callus induction from leaf explants

The problems phenolic oxidations was encountered upon culturing of various coffee explants

Such as leaf, leaf stalk, node, inter node, apical and auxiliary buds for callus induction. In order to overcome the problem of browning, the media were supplemented with ascorbic acid (20 mg/l or L-ysteine HCl 10 mg/l as an antioxidant to reduce the oxidation of phenolic compounds, hence to prevent the browning of tissue and callus.

The present results showed that out of the different combinations of auxins (0.2, 0.4, 0.6, 0.8 mg/l and 1 mg/l of IAA, NAA, IBA and 2, 4D) and cytokinins (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mg/l) of BAP or kinetin employed in a separate experiment for callus induction from leaf explants, only six auxin/cytokinin combinations (1mg/l BAP and 1mg/l 2,4D; 1.5 mg/l BAP and 0.5 mg/l 2,4D; 3 mg/l BAP and 0.2 mg/l 2,4D; 1.5mg/l BAP and 0.2 mg/l 2,4D; 4.5mg/l chinetin and 0.5 mg/l NAA; and 4.5 mg/l chinetin and 0.2 mg/l NAA induced callus formations. Callus induction occurred in the range of three to four weeks after the inoculation of the explants. Each callus initiated on specific culture medium was sub cultured on identical but fresh medium at an interval of four weeks after callus initiation. Attempts made to transfer the calli onto different media apart from half strength MS media of their respective induction medium resulted in the die back of the calli.

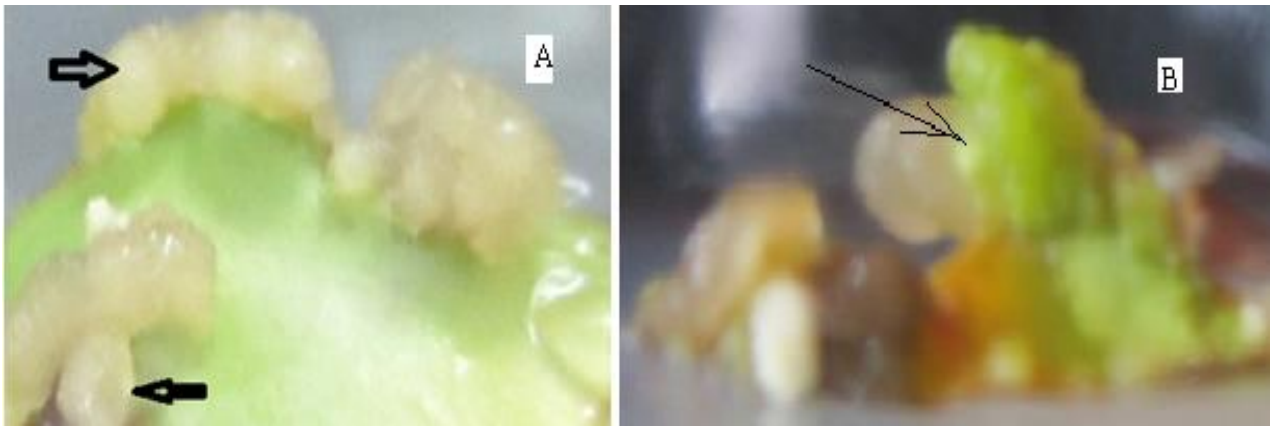

Fig 1: Callus induction "A" on MS medium supplemented with 4.5 mg/l chinetin and 0.2 mg/l NAA, and somatic embryo induction "B" on half strength MS medium supplemented with 3 mg /l chinetin and 0.2 mg/l NAA, from *C. arabica* leaf explants

Effects of cytokinins (BAP and Kin) and auxins (2,4D and NAA)

The BAP/2,4D growth hormone combinations (table1) produced higher callus biomass than the Kin/NAA growth hormone combinations, indicating relatively higher candidacy of BAP/2,4D hormonal combinations in media for callus initiation from leaf explants of *C. arabica* L

Table 4. Summary of sample fresh weight, dry weight and moisture contents of calli initiated from leaf explants on MS media supplemented with varying concentrations of specific cytokinin/auxin combinations.

Table 4

Categories	FW gm	DW gm	% moisture content
1 mg/l BAP and 1 mg/l 2,4D	2.35	0.188	92
1.5 mg/l BAP and 0.5 mg/l 2,4D	1.14	0.103	91
1.5 mg/l BAP and 0.2 mg/l 2,4D	1.18	0.130	89
3 mg/l BAP and 0.2 mg/l 2,4D	1.17	0.117	90
4.5 mg/l kin and 0.5 mg/l NAA	1.04	0.094	91
4.5 mg/kin and 0.2mg/ NAA	1.12	0.090	92

Table 5: Influence of IBA on number of roots, root length, of *C. arabica* micro-shoots initiated from leaf explants through indirect embryogenesis

IBA Mg/l	Number of roots (cm)	Root length (cm)	Callusing
0.0	0.0d	0.0d	-
1.0	1.5c	2.0c	+
2.0	3b	3.5b	++
3.0	6.0a	6.2a	+++

Having different letters are significantly different according to the least significant difference (LSD) at 0.05 level of probability. (-) = no callus, (+. ++ and +++) = (callus with 2-4, 4-6 and 8-10 mm) in diameter, respectively.

Table 6. Influence of NAA on number of roots, root length, of *C. arabica* micro-shoots initiated from leaf explants through indirect embryogenesis

Table 6

NAA Mg/l	Number of roots (cm)	Root length (cm)	Callusing
0.0	0.0d	0.0d	-
1.0	2.4c	2.3c	+++
2.0	3b	3.5b	++++
3.0	6.0a	6.2a	++++++

Having different letters are significantly different according to the least significant difference (LSD) at 0.05 level of probability. (-) = no callus, (+++. ++++ and +++++) = (callus with 4-6, 8-10 and 12-14 mm) in diameter, respectively.

The present study was performed to optimize tissue culture protocol for *C. Arabica* L. samples obtained from India. Investigation was held to select the best auxin//cytokinin combinations for indirect somatic embryogenesis from leaf explant of *C. arabica*. The results reveal that the leaf explants exhibited varying responses to different concentrations of auxins and cytokinin combinations.

The presence of phenolic compounds causing the deaths of explants is an important issue to be seriously considered in tissue cultures of perennial woody plants (Compton and Preece, 1986). Addition of antioxidants or reducing agents like ascorbic acid in the medium or before surface sterilization helps to reduce the redox potential and hence minimizes the oxidation reduction reaction (Marks and Simpson, 1990) [9]. Due to the oxidation of externally released polyphenols, explants as well as the nutrient medium become brown and result in the failure of response of explants to in vitro culture.

The onset of tissue browning has been reported to be associated with changes in patterns of amino acids content, ethylene production, and accumulation of starch (Linofers *et al.*, 1990). In the present investigation the use of ascorbic acid and L-Cysteine HCl in callus/somatic embryo inductions and shoot regenerations, respectively played significant roles in minimizing the effects of phenolic oxidations.

In the present investigation, only leaf explants responded to

callus induction media. However, the leaf explants have not significantly responded to the majority of the auxin and cytokinin combinations tested. This relatively low response might be due to the source of the explants (greenhouse versus *in vitro* germinated and grown coffee plants). Santana *et al.* (2007) [18] reported that the explants from plants cultured *in vitro* are more responsive than those from greenhouse or field plants. In addition, the pretreatment of the plants with auxins, mainly NAA, also made their explants more responsive to callus or somatic embryo development. Furthermore, the position of the leaves on the plant was very important, as the first two pairs of leaves did not show any calli or embryogenic response, and the explants coming from the distal part of the leaf were less responsive than those coming from the basal part of the leaf (Santana *et al.* (2007) [18]. In the present report, no distinction was made in the position of leaves during leaf explants sampling and their subsequent inoculation. This may also have contributed to lower or absence of callus induction response of *C. arabica* leaf explants to many of the growth hormone combinations tested. Moreover, Molina *et al.* (2002) [10] reported that coffee callus induction also depends on age and genotype of the explants. The genotype effect on callus induction ability was also reported previously in sugarcane (Gandonou *et al*., 2005) [16].

Among the different types and concentrations of auxins and cytokinins incorporated in the medium, 2, 4D in combination of BAP was most useful for higher callus induction and proliferation as compared to other auxin/cyokinin combinations that initiated callus formation table 4). In contrast to the present result, Söndahl and Sharp (1977) [21] reported that 2,4D in combination with kin has higher potential for callus induction and proliferation.

Micro-shoots were rooted on half-strength MS medium. IAA had a significant effect on rooting of *in vitro* grown coffee micro-shoots. Increasing IAA to 3.0 mg/l gave significantly the highest number of roots, root length and shoot height. No callus formation was observed on the basal part of the micro-shoot when 1.0 mg/l IAA was used. On the other hand, the 2.0 or 3.0 mg/l IAA showed small callus formation on the basal part of the micro-shoots. Table5 indicates the effect of IBA on rooting of coffee micro-shoots. Higher number of roots, root length and shoot height were obtained with 3.0 mg/l IBA. Table6 shows the effect of different concentrations of NAA on rooting parameters of coffee. It is clear that NAA was the least suitable as it produced much callus and failed to promote good rooting parameters in comparison to other auxins. Rooting parameters significantly decreased at 3.0 mg/l NAA. This could be due to large callus mass (10-12 mm) around the basis of micro-shoots. Our results are in agreement with the finding of Kahia and Owuor (1990) who rooted coffee micro-shoots on half-strength MS media supplemented with 2.0 mg/l NAA.

Rooted plantlets showed different survival percentage according to plant growth regulator used, Table7. *C. arabica* plantlets rooted on IAA or IBA gave complete survival percentage (100%) under acclimatization. The lowest survival percentage was found when NAA was used. This could be due to the presence of short roots and large callus mass around the basis of rooted plantlets in case of NAA. Plants that were transferred to the greenhouse after acclimatization had 86% survival and reached about `8 cm length after two and half months.

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References

1. Ascanio ECE, Arcía MM. Haploids from anther culture in Coffea arabica L, Bogotá, Colombia; In: International Congress of Plant Tissue Culture, Tropical Species. 1987, 68.
2. Carneiro MF, Ribeiro TMO. *In vitro* micropropagation of C. Arabica L. CV, Caturra by means of axillary buds, Broterica. 1989; 85:139-152.
3. Carvalho A. Principles and practice of coffee plant breeding for productivity and quality factors: *Coffea arabica*. In: Fewerda FP, Wit F. eds. Coffee: agronomy, London: Elsevier Applied Science. 1988; 4:129-165.
4. Cros J, Combes MC, Trouslot P, Anthony F, Hamon S, Charrier A, *et al.* Phylogenetic relationships of *Coffea* species: new evidence based on the chloroplast DNA variation analysis. Mol. Phylogenet. Evol. 1998; 9:109-117.
5. Dublin P, Enjalric F, Lardet F, Carron MP, Trolinder N, Pannetier C. Estate crops. 1. Coffea species. In: Micropropagation (eds P.C. Debergh and R.H. Zimmerman). Kluwer Academic Publ. 1991, 337-361.
6. Gandonou Ch, Abrini J, Idaomar M, Senhaji SN. Response of sugarcane (*Saccharum sp*.) varieties to embryogenic callus induction and *in vitro* salt stress. Afr. J. Biotechnol. 2005; 4(4):350-354.
7. Lanaud C. Production of *Coffea canephora* plantlets by somatic embryogenesis obtained by in vitro culture of ovules. Café Cacao Thé. 1981; XXV:231-236.
8. Lindofers A, Kuusela H, Hohtola A, Kupila-Ahvenniment S. Molecular correlates of tissue browning and deterioration in scot pine calli. Biologia of plant. 1990; 32:171-180.
9. Marks T, Simpson S. Reduced phenolic oxidation at culture initiation following the exposure of field grown stock plants to darkness or low levels of irradiance. Journal of Horticultural Science. 1990; 65:103-111.
10. Molina MD, Aponte EM, Cortina H, Moreno G. The effect of genotype and explant age on somatic embryogenesis of coffee. Plant Cell Tiss. Org. Cult. 2002; 71(2):117-123.
11. Monaco LC, Söndahl MR, Carvalho A, Crocomo OJ, Sharp WR. Application of tissue culture in the improvement of coffee. In: Applied and Fundamental Aspects of Plant Cells, Tissue and Organ Culture. (Eds): Reiaert J, Bajaj YPS. Narosa Publ. House, India, 1995.
12. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 1962; 15:473-497.
13. Nassuth A, Wormer TM, Bouman F, Staritsky G. The histogenesis of callus in *Coffea canephora* stem explants and the discovery of early embryoid initiation. Acta Bot. Neerl. 1980; 29:49-54.

14. Orozoco-Castillo F, Chalmers KJ, Waugh R, Powell W. Detection of genetic diversity and selective gene introgration coffee using RAPD markers. Theor. Appl. Genet. 1994; 87:934-940.
15. Quiroz-Figueroa FR, Fuentes-Cerda CFJ, Rojas-Herrera R, Loyola-Vargas VM. Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of Coffea arabica. Plant Cell Rep. 2002a; 20:1141-1149.
16. Quiroz-Figueroa FR, Méndez-Zeel M, Larqué-Saavedra A, Loyola-Vargas VM. Picomolar concentrations of salycilates induce cellular growth and enhance somatic embryogenesis in Coffea arabica tissue culture. Plant Cell Rep. 2002b; 20:679-684.
17. Raghuramulu Y, Purushotham K, Sreenivasan MS, Ramaiah PK. *In vitro* regeneration of Coffee plantlets in India. J Coffee Res. 1987; 17:57-64.
18. Santana-Buzzy N, Rojas-Herrera R, Rosa M. Galaz-Ávalos, Ku-Cauich RM, Mijangos-Cortés JR, Gutiérrez-Pacheco J, *et al.* Advances in coffee tissue culture and its practical applications, *In Vitro* Cell. Dev. Biol-Plant. 2007; 43:507-520.
19. Shibli RA. Jordan tissue culture industry makes great strides. Diversity. 1995; 11-77.
20. Shibli RA, Ajlouni M, Jaradat A, Aljanabi S, Shatnawi M. Micropropagation of wild ear (Pyrussyriaca). Hort. Sci. 1997; 972:1-6.
21. Söndahl MR, Sharp WR. High frequency induction of somatic embryos in cultured leaf explants of Coffea arabica L. *Z. Pflanzenphysiol*. 1977; 81:395-408.
22. Sreenath HL, Shanta HM, Babu KH, Naidu MM. Somatic embryogenesis from integument (perisperm) cultures of coffee. Plant Cell Rep. 1995; 14:670-673.
23. Staritsky G. Embryoid formation in callus tissues of coffee. Acta Bot. Neerl. 1970; 19:509-514.