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In vitro regeneration of shoots from apical and auxiliary bud explants of *Coffea arabica* L

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Abstract
The aims of the present investigation were to optimize tissue culture protocol for *C. arabica*, to identify the best explants and growth hormone combinations and their respective concentrations suitable for *Coffea Arabica* L. *in vitro* multiplication. In a present investigation, surface sterilized apical and auxiliary bud explants were cultured on MS media for shoot regeneration and multiplication. The media were prepared in four different combinations of auxins and cytokinins supplemented with 30 g/l sucrose, 4 g/l agar and 10 mg/l L-Cysteine HCl, L-Cysteine HCl was used to control the oxidation of phenolic compounds. A similar experiment was repeated under dark condition by covering the culture vessels with black polyethylene plastic bags to study the effect of darkness on growth response of the explants. Each treatment consists of 5 replicates in a completely randomized design. The results showed that both bud explants regenerated shoots in all the four media employed, and shoots regenerated from apical bud explants were vigorous than those regenerated from auxiliary bud explants. Results are discussed from *C. arabica* L. tissue culture protocol optimization point of view.

Keywords: Apical buds, auxiliary buds, auxins *C. Arabica* L., cytokinins

Introduction
Coffea Arabica L. is a species that originates from the Ethiopian highlands (Cros *et al*, 1998) [4]. 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) [1]. 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) [13].

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].

Propagation by tissue culture technique could provide a viable alternative to the traditional methods of coffee propagation. Tissue culture method permits the production of relatively uniform plants on a massive scale in a short period and with narrow genetic base than is possible under the conventional methods. 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*., 1995) [14].

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) [14]. 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) [14]. 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) [16].. Therefore, the aims of this investigation were to optimize tissue culture protocol for *C. Arabica* L., to identify the best explants and growth hormone combinations and their respective concentrations suitable for *Coffea Arabica* L. in vitro regeneration of shoot from apical and auxiliary buds

Materials and Method

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).

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

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 5 ºC for later use (table2-3). Each MS stock solution needs to be renewed after each month.

Table 2: 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

Table 3: 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 growth hormones (Auxins and Cytokinins) stock solutions

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 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 ºC 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 apical and auxiliary bud explants

Surface sterilization of coffee apical and auxiliary bud explants from green house were accomplished by careful selection of undamaged and healthy buds 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 shoots from apical and auxiliary bud explants

The surface sterilized apical and auxiliary bud explants were cultured on a separate MS media for shoot initiation and multiplication. The media were prepared in four different combinations of auxins and cytokinins supplemented with 30 g/l sucrose, 4 g/l agar and 10 mg/l L-Cysteine HCl (table4). The growth regulators used were 1mg/l of auxins (IAA, IBA, NAA and 2,4D) and 0.1 mg/l of cytokines (kinetin). L-Cysteine HCl was used to control the oxidation of phenolic compounds. A similar experiment was repeated under dark condition by covering the culture vessels with black polyethylene plastic bags to study the effect of darkness on growth response of the explants. Each treatment consists of 5 replicates in a completely randomized design.

The pH of the medium was adjusted at 5.8 and autoclaved at 121 ºC for 20 minutes. Approximately 25 ml of medium was dispensed in glass vessel with 10 replicates each. After inoculation of surface sterilized apical and auxiliary bud explants, the culture were maintained in growth room at 26 ºC ± 2 ºC. for shoot initiation and multiplication. The cultures were observed for growth or contamination every day after inoculation and incubation.

Table 4: Different MS media used for shoot induction from apical and auxiliary bud explants of *C. Arabica* L.

Categories	Control	A	B	C	D
	-	IAA	IBA	NAA	2,4D
	-	1 mg/l	1 mg/l	1 mg/l	1 mg/l
Kinetin	-	0.1 mg/l	0.1 mg/l	0.1 mg/l	0.1 mg/l
L-Cysteine HCl	-	10 mg/l	10 mg/l	10 mg/l	10 mg/l

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 compost mixed 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±2 ºC, and were irrigated with 1/4th 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

In all shoot regenerating media employed for shoot induction from apical and auxiliary bad explants, calli were formed at the base of the regenerated shoots. Shoot regeneration was not observed from apical and auxiliary bud explants in MS medium (control column table4) not supplemented with growth hormone plus or minus L-cysteine HCl, and the explants survived for two weeks and eventually died in the third week. Both apical and auxiliary bud explants showed growth in all the four media (table4). However, apical buds (figure1) exhibited vigorous induced shoot than shoots induced from auxiliary buds (figure2).

A similar experiment was repeated under dark condition by covering the culture vessels with black polyethylene plastic bags to study the effect of darkness on growth response of the explants showed no significant shoot induction difference when compared to the shoots induced under light conditions for the first two weeks of inoculations. After the third week the explants inoculated and kept under dark condition began to deteriorate and finally died after the fourth week of inoculation.

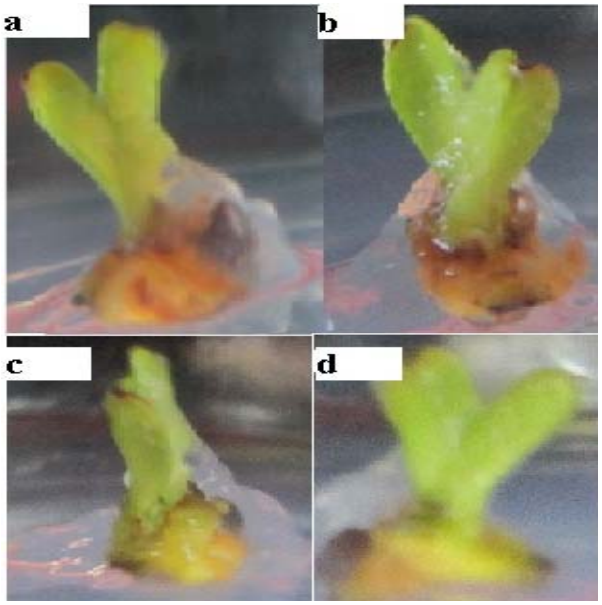

Fig 1: Shoot induction from apical bud explants. Panel "a" is in MS medium containing 1mg/l IAA and 0.1 mg/l kinetin, Panel "b" is in MS medium containing 1mg/l IBA and 0.1 mg/l kinetin, Panel "c" is in MS medium containing 1mg/l NAA and 0.1 mg kinetin, and Panel "d" is in MS medium containing 1mg/l 2, 4D and 0.1 mg/l kinetin. All media were supplemented with 10% L-Cysteine HCl.

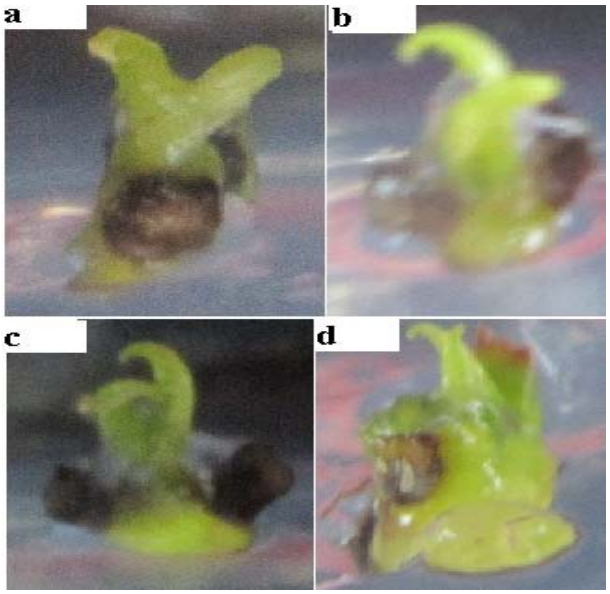

Fig 2: Shoot induction from auxiliary bud explants. Panel "a" is in MS medium containing 1mg/l IAA and 0.1 mg/l kinetin, Panel "b" is in MS medium containing 1mg/l IBA and 0.1 mg/l kinetin, Panel "c" is in MS medium containing 1mg/l NAA and 0.1 mg kinetin, and Panel "d" is in MS medium containing 1mg/l 2, 4D and 0.1 mg/l kinetin. All media were supplemented with 10% L-Cysteine HCl.

Discussion

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 explant sources for *C. arabica* in vitro multiplication. The results reveal that different explants sources 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) [3]. 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) [10]. 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) [9]. 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 results of shoot regenerations from apical and auxiliary bud explants shoots originated from apical bud explants (fig.1) were found to be more vigorous than shoots originated from auxiliary bud explants (fig.2). Ismail *et al.* (2003) [8] also reported that apical buds are more vigorous in culture growth than auxiliary buds. On the contrary, Sondal *et al*. (1985) [15] reported that the rate of growth of auxiliary shoots improves and increases in tissue culture compared to the growth of apical shoots. Dodds &Roberts (1986) [5] suggested that the apical bud explants showed best growth than the auxiliary bud explants because it receives minimum wound and injury, thus secretes minimum phenolic compounds. Polyphenolases stimulated by injury oxidizes these phenolic substances two growth inhibitory dark color compounds. He further explained that during collection of explants, wounds and cuts initiate the oxidation process in coffee plant. Therefore, special precautions should be taken for preventing the accumulation of toxic products. Tissues containing relatively high concentrations of phenolic compounds like coffee plants are difficult to culture (Ismail *et al*., 2003) [8]. The brown color that frequently develops in cultures of coffee is due the formation of quinones, which are well known to be toxic to microorganisms and inhibitory to plant's cellular growth (Monaco *et al*., 1995) [11]. In the present investigation, incorporation of L-Cysteine HCl minimizes the formation phenolic compounds and results in the growth of apical and auxiliary bud explants in all the media employed. The regeneration of shoots from apical and auxiliary bad explants under dark condition to study the effect of darkness on growth response of the explants showed no significant difference from the ones conducted under light conditions. This result is in disagreement with the report of Ismail *et al.* (2003) [8] in which he stated that cultures incubated in the dark showed best results than the cultures incubated in light. This was further supported by Monaco *et al.* (1995) [11] who explained that maintenance of culture in the dark promotes growth because illumination is stimulatory to the production of phenolics. Flick (1983) [7] has also described that phenolic compounds are produced when grown in the light than in the dark. Contrary to these reports our present result showed that cultures kept under dark conditions survived for the first three weeks of incubation and then started deteriorating and finally terminated in death. This implies that the significance of light for shoot growth outweigh its side effects.

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