Evaluation of chemical and physical parameters for callus induction from anther cultures of tea (*Camellia sinensis* (L.) O. Kuntze)

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Abstract

Tea belongs to the family Theaceae. Next to water, tea is the most consumed non-alcoholic beverage in the world. Tea plant synthesizes a lot of medicinally important secondary metabolites such as caffeine, theanine, catechin, primeverosides, linalool, geraniol and lutein. One basic requirement for their utilization at commercial level is the constant availability with standard quality. However, inconsistent environmental and genetic factors, and high out-breeding nature of the plant contribute to the uneven metabolite production. Plant cell and tissue culture offer solutions to overcome these problems where by manipulation of culture conditions, constant availability of cell biomass can be made available as an alternative source for these metabolites, irrespective of the seasonal constraints.

By keeping in view the above problem, the anther cultures, bearing early-to-late uninucleate stage of microspores, were raised to induce callus. Various chemical and physical treatments like growth regulator combinations, sucrose concentration and temperature pre-treatments to cultures were given to induce sustained and massive callus proliferation. In the absence of growth regulators (Basal Media) the response was nil. However, in presence of two auxins (NAA and 2,4-D) and at least one cytokinin (BAP/Kinetin), callus response was substantially improved. Control (25℃) and Cold temperature (5℃) treatments showed encouraging results for callusing while heat treatment was entirely unfavorable. Similarly, high sucrose concentration (9%) was found suitable for callusing. Through repeated subculturing on the same parent media, it was possible to achieve moderately growing, green and healthy calli in every 5-6 weeks. Further experiments are in progress to scale up the cell biomass.

Keywords: *Anther culture – Callus – Camellia sinensis—Tea*

1. Introduction

Camellia sinensis (L.) O. Kuntze , commonly known as " Tea", belongs to the family Theaceae. Tea was originated from South-East Asia i.e. intersection between 29° N (latitude) and 98° E (longitude) near the source of the Irrawaddy river at the confluence of North-East India, North Burma, South-West China and Tibet provinces[16]. It is one of the most consumed non-alcoholic beverages in the world next to water and is a major cash crop in a number of developing countries including India. Despite occupying only around 16% of the total tea growing areas of the world, India ranks first as the producer, consumer and exporter of tea and, hence, it plays an important role in building the national economy. Tea plant synthesizes more then 700 chemical constituents, among which flavanoides, amino acids, vitamins (C, E, and K), caffeine and polysaccharides are required for health[7]. The available *Camellia* varieties are genetically highly heterozygous and, thus, are responsible for uneven metabolite production. At this juncture, biotechnology offers an opportunity to exploit the cells, tissues or organs by growing them in vitro and to get desired compound at a constant rate by selecting suitable best cell lines, independent of geographical and seasonal variations.

In this context, anther culture presents a number of potential advantages over other explant cultures, mainly, in relation to in vitro selection strategies and genetic studies. Wall of the anther constitutes diploid sporophytic tissue while microspore population within the anther are haploid in nature. Thus, the proliferation of cells from such tissues may provide an equal opportunity for the screening of somaclonal as well as gametoclonal variation at one go. Furthermore, mutations which are usually recessive and do not express themselves in the presence of their dominant alleles in heterozygous, cross pollinating trees like tea; in haploid cells it can be easily expressed because they have a single set of genes. More so, chromosomes of these haploid cells can be duplicated by colchicine treatment to obtain homozygous diploid lines in a single generation. As a result, callusing, if obtained, from anther cultures of *C. sinensis*, would be potentially valuable for the screening of pharmacologically important secondary metabolites. These single cell lines have several enticements over whole plant processing which offers several advantages including efficiency, product quality and yield, space requirement, and uniformity of cell populations and products, irrespective of environmental conditions. In view of that, the present study is aimed to develop cell biomass from important Tocklai Vegetative (TV_1) cultivar of *C. sinensis* by manipulating in vitro physical and chemical conditions.

2. Materials and Methods

2.1. *Plant Material and Establishment of Aseptic Cultures*

The buds from the first flush of flowers, in the months from November-January, were used to initiate anther cultures. The stage of microspore development was determined by acetocarmine squashes. Young flower buds of the size 3-4mm, bearing microspores at the early-to-late uninucleate stages, were collected between 6.30 to 7.30 AM from the cultivar TV_1 and brought to the laboratory. The buds were taken to the laminar-air-flow cabinet and further operations were carried out under aseptic conditions. After surface sterilizing with 0.1% solution of $HgCl_2$ for 7 min, followed by 3 washings with sterile distilled water (SDW), the buds were dissected under a Stereo-microscope, using pre-sterilized Petriplates, forceps and fine needles. The damaged anthers, if any, were discarded, and the filament was gently removed. Twenty anthers were cultured in 60mm x 15mm pre-sterilized, disposable Petriplates containing 10ml of MS medium with or without growth regulators. The Petriplates were sealed with parafilm.

2.2. *Culture Media*

MS^9 basal medium was used throughout the studies to raise anther cultures. They were variously supplemented with a range of growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), N^6-furfuryl adenine (Kinetin) or 6-benzylaminopurine (BAP), either individually or in combinations. The media contained 3% sucrose and were gelled with 0.8% agar (Qualigens, India). To study the effect of sucrose concentration on anther callusing, two higher concentrations of sucrose, 4% and 9% were also tested.

Analytical grade (AR) chemicals and glass-distilled water were used to prepare stock solutions and culture media. Stock solutions of macronutrients (×10), micronutrients (×20), iron (×20) and vitamins (×20) were made separately and stored at 4°C till further use. The stock solutions of growth regulators were prepared at a concentration of 1×10^{-3}M and stored in refrigerator. Myo-inositol and sucrose were weighed and directly added to the culture medium at the time of media preparation. After adjusting the pH to 5.8, by using 0.1N HCl or 0.1N NaOH, the medium was dispensed into Erlenmeyer flasks containing weighed quantity of agar. The media were autoclaved and allowed to cool down to ca 50°C

before dispensing into 60mm pre-sterilized Petriplates (10ml medium per plate) under aseptic conditions.

2.3. *Physical Parameters*

The cultures were generally incubated at 25 ± 2°C and 50-60% relative humidity. To understand the effect of light on callus induction, the cultures were incubated in diffuse light (1000-2000lux) with 16h photoperiod provided by cool day light fluorescent tubes (Philips TL 40W) as well as in continuous darkness. The level, time of application and duration of temperature pre-treatments were also examined on anther callusing. The cultures were maintained either continuously at 25°C (control) or pre-treated for 5 days at low (5°C) and high (33°C) temperatures before being shifted to control temperature (25°C). In one of the experiments, even the flower buds were subjected to low and high temperature regimes for 5 days.

2.4. *Observation of Cultures*

For each treatment, 100 anthers were raised. The cultures were observed periodically, and the morphological changes were recorded at weekly intervals or whenever necessary. Final observations were taken after 6 weeks. The number of explants showing callus formation was recorded and is expressed as percent response.

3. Results and Discussion

3.1. *Callus Induction*

For callus induction, anther cultures of the cultivar TV_1, at early-to-late uninucleate stage of microspores, were exposed to various chemical and physical treatments like, growth regulator combinations, sucrose concentration, temperature pre-treatments and light vs dark incubation of cultures.

3.1.1. *Hormonal Treatments*

In the absence of a growth regulator (control) the response was negligible. The combined presence of 2,4-D and NAA induced some callusing but the response was poor. However, in presence of two auxins (NAA and 2,4-D) and at least one cytokinin (BAP/Kinetin), callus response was substantially improved. On MS + 2,4-D (1µM) + NAA (1µM) + BAP (5µM), 76.6% anthers callused, and the callus growth was good. Compared to this, when BAP (5µM) was replaced with Kinetin (1µM), only 52.5% cultures responded for callusing.

Most of the anthers were swollen after one week of culture initiation (Fig. 1A). The anther sacs burst open after 5-6 weeks and start releasing shiny, white and transparent callus from inside the anther locules along with the granulated callus from the periphery of the anther (Fig. 1B).

3.1.2. Effect of *Sucrose Concentration*

MS medium normally contains 3% sucrose (control). Two higher concentrations of sucrose at 4% and 9% were tested to study their effect on callus induction from anthers. For all the treatments, medium was supplemented with 2,4-D, NAA and BAP/Kinetin. Sucrose at 9% concentration was distinctly better showing excellent callus growth while no growth was observed at either 3% or 4% sucrose concentration.

3.1.3. Effect of *Temperature Pre-treatments*

The level, time of application and duration of temperature pre-treatments are known to have significant effect on anther callusing in several systems. Therefore, flower buds as well as cultured anthers of the cultivar TV_1 were exposed to 5ºC and 33ºC temperature pre-treatments for 5 days to induce callusing from anthers. Besides, cultures were also maintained continuously at 25°C (control) temperature.

Temperature pre-treatments to buds were found to be insignificant. Whereas, response from anthers exposed to cold temperature pre-treatment (5ºC) was comparable to that of the control (25ºC), heat (33ºC) treatment was proved to be insignificant. For MS (9% sucrose) + 2,4-D (1 µM) + NAA (1µM) + BAP (5µM), control (25ºC) temperature was better than cold treatment where 76.6% and 64% cultures responded, respectively, for callusing. In contrast to this, on MS (9% sucrose) + 2,4-D (1 µM) + NAA

(1µM) + Kinetin (1µM) medium, higher percentage (61.7%) of cultures showed callusing at cold temperature than at control (52.5%) (Fig. 2).

3.1.4. *Effect of Light Incubation*

Light was not found to be mandatory for induction of cell divisions in anthers. Anther cultures incubated continuously in dark gave more than four times better result than those maintained throughout in light (Fig. 3).

3.2. *Callus Multiplication*:

The calli (measuring ca. 5mm×5mm) were subcultured on the original media of same combination after 6 weeks of induction. Irrespective of the origin of the calli, browning of cultures and emergence of microbial contamination was the major problem in the second sub-cultures; surprisingly, none of the primary cultures showed any contamination. Thus, it is suggested that cultures might had contained endogenous microbial colonies, which were hidden in primary cultures but reappeared in second sub-cultures with the availability of fresh nutrition. However, through repeated subculturing on the same parent media, we could be able to achieve moderately growing, green and healthy calli in every 5-6 weeks (Fig. 1C). Cytological squash preparation of these calli showed presence of haploid as well as diploid cells indicating their origin from pollen as well as wall tissues. Efforts are now being concentrated to increase the cell biomass and to receive shoot regeneration as well from these calli.

Discussion

The available *Camellia* varieties are genetically highly heterozygous. In this genus, frost and pathogens cause serious damage that result in drastic annual economic losses. So far, most of the reports on application of in vitro techniques have been concerned with the establishment of protocol for micropropagation from juvenile and adult materials of mainly China, Japan and Srilanka[1,3,5,10,12,13,14,15]. There are very few reports on in vitro culture of tea varieties grown in India[2,4,6,8]. However, in majority of the above reports mostly diploid tissues like, leaf, cotyledon or nodal segments were commonly used for micropropagation. Chen and Liao[3] and Raina and Iyer[11] raised anther cultures as a strategy for plant improvement for *C. sinensis*. In the present study, anther cultures were raised with the aim to develop

large-scale cell biomass for their later utility to produce pharmacologically important secondary metabolites. In this reference, anther cultures present a number of potential advantages by offering the opportunity for screening of gametoclonal variation in addition to somaclonal variation, simultaneously. Secondly, in most of the reports, major problems encountered in tea are phenolic exudation from explants and microbial contamination of cultures, causing severe losses at every stage of microprogation (see Mondal et al[7]). The present study, suggested the repeated subculturing of calli to overcome the problem of microbial contamination and to obtain sustained and massive callus proliferation. In conclusion, the method discussed in the present report has the potential to be used to produce large biomass of tea, in vitro.

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Figure Legends

Figure 1:

A. Anther at culture showing swelling and longitudinal fissure after one week of culture initiation.

B. 6-week old anther culture. Anther locules burst open releasing transparent callus (arrow marked); Note the callusing from the surface of the anther as well.

C. Multiplying green and healthy callus after repeated subcultures.

Figure 2: Percent callus induction in anthers, incubated in dark, at various temperature pretreatments. Growth Period: 6 weeks; Basal Medium: MS (with 9% sucrose).

Figure 3: Effect of Light and Dark Incubation on callus induction in anthers of TV_1 at 25˚C. Growth Period: 6 weeks; Basal medium: MS (with 9% sucrose).

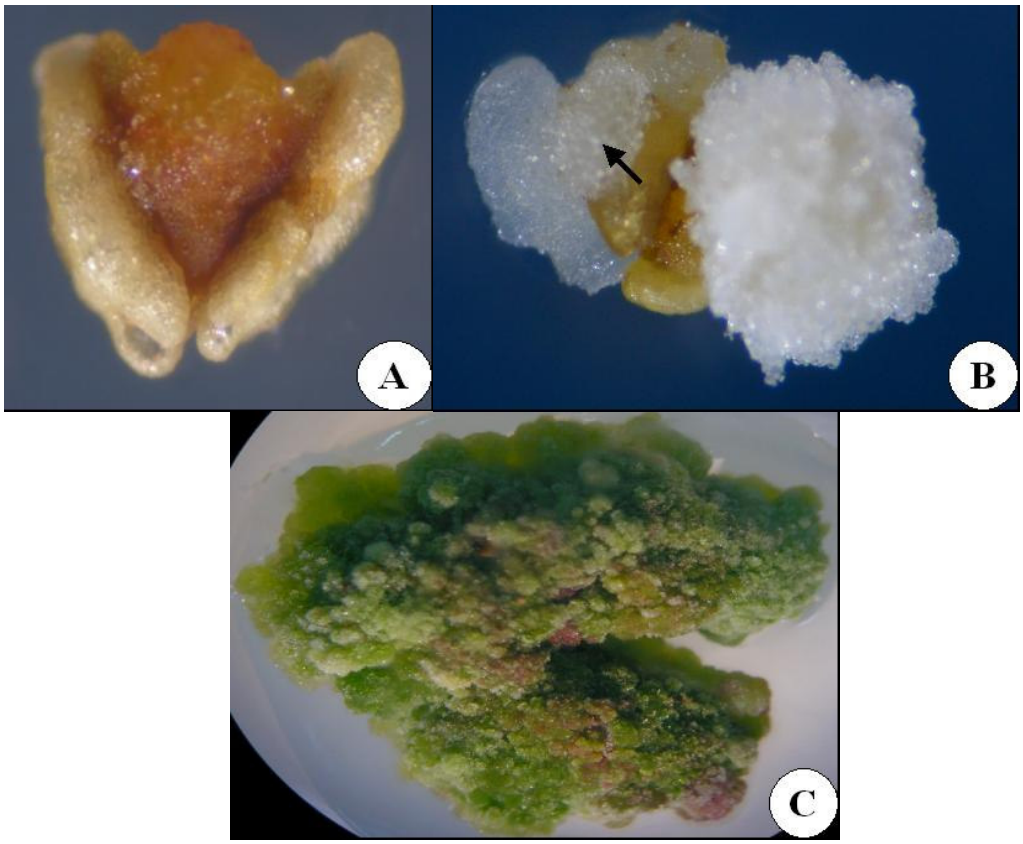

Figure 1

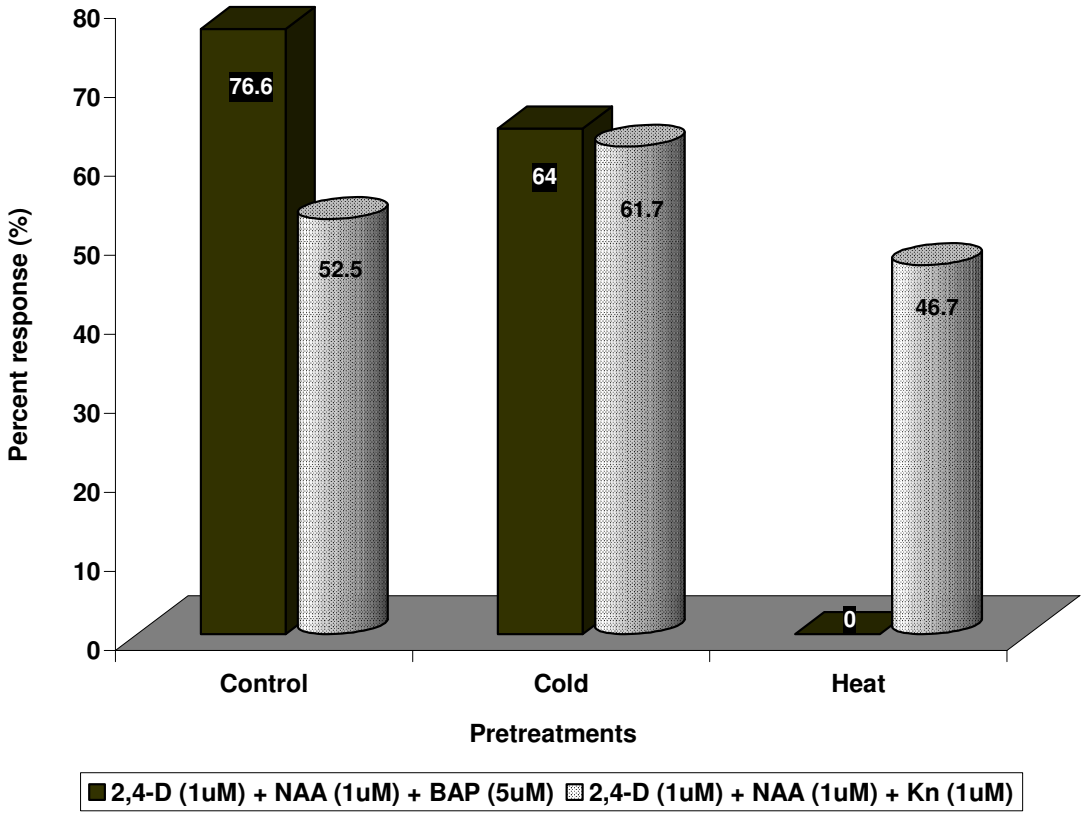
80
70
60
50
40
30
20
10
0
Percent response (%)
76.6
52.5
64
61.7
0
46.7
Control
Cold
Heat
Pretreatments
2,4-D (1uM) + NAA (1uM) + BAP (5uM)
2,4-D (1uM) + NAA (1uM) + Kn (1uM)

Figure 2

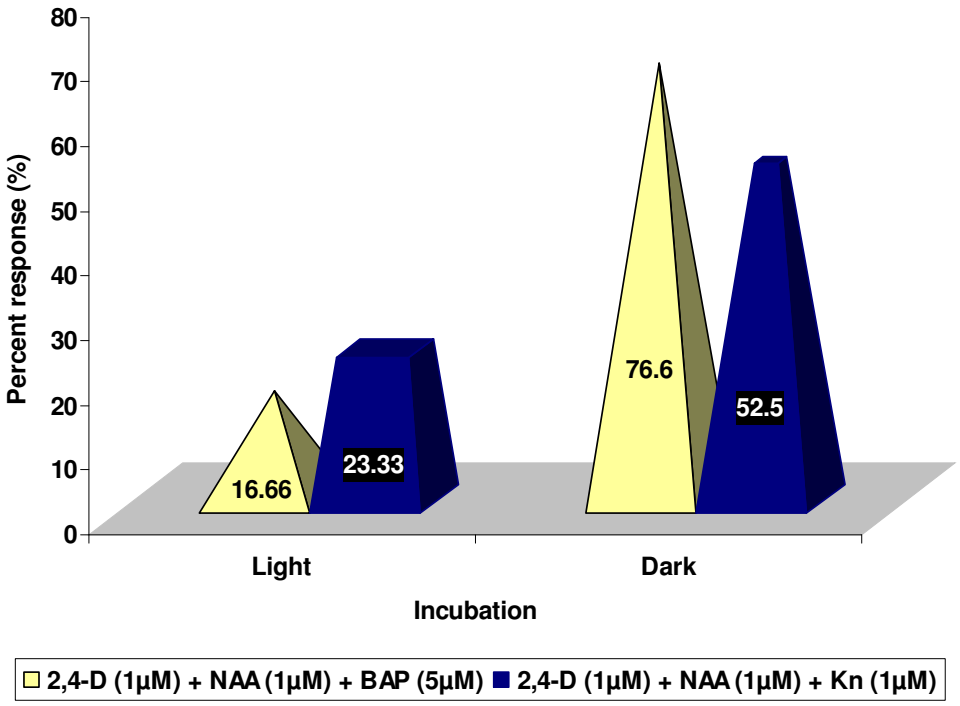
80
70
60
50
40
30
20
10
0
Percent response (%)
16.66
23.33
76.6
52.5
Light
Dark
Incubation
2,4-D (1µM) + NAA (1µM) + BAP (5µM)
2,4-D (1µM) + NAA (1µM) + Kn (1µM)

Figure 3