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OPTIMIZATION AND ESTABLISHMENT OF *ACTINIDIA DELICIOSA* CELL SUSPENSION CULTURE TO STUDY THE BIOTRANSFORMATION FOR THE PRODUCTION OF NOVEL COMPOUNDS

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Abstract

A highly efficient protocol for the induction of callus and establishment of cell suspension culture was developed for *Actinidia deliciosa*. Germination of *Actinidia* seeds were carried out on full strength MS medium without any growth hormone and the leaves from *In vitro* grown plantlets were used for the induction of callus. Maximum amount of friable calli were obtained on full strength MS medium supplemented with 1.0 mg/L IBA (Indole-3-butyric acid) and 0.5 mg/L BAP (6-Benzyl amino purine) with the 16 hrs illumination period. Cell suspension cultures were established using MS medium with higher amount of IBA (2.0 mg/L) and BAP (0.5 mg/L), by rotating the culture flasks at 110 rpm on a gyratory shaker. Biotransformation ability of the suspension culture of *Actinidia deliciosa* was also determined by adding (-)-Ambrox to the cultures as a substrate. Six transforms were isolated after 15 days of incubation while two of them were found novel.

Introduction

The Kiwifruit (*Actinidia deliciosa*) is a large, woody, deciduous vine, native to the Yantze Valley of China (Anon., 2004). The plant is well known for its great medicinal importance and extracts from different parts of the plant are used for treating mange in dogs and reported with laxative effect (Rush *et al*., 2002; Collins *et al.*, 2003). The presence of glutathione in the fruit juice may account also for the reduction in mutagenesis (Tanimoto & Oritani, 1977; Ferguson *et al*., 2004).

The chemical modifications carried out by living cells, organs or enzymes are known as biotransformation which has a great potential to generate novel products of high medicinal importance. These compounds can be chemically synthesized but the process might be of high cost and labour intensive and results in the production of large quantities of waste organic solvents which are harmful to the environment (Hamada & Furuya, 1996; Schulze & Wabbolts, 1999). Biotransformation can be carried out using variety of living cells including microbial (Hanson & Truneh, 1996; Ward & Singh, 2000), fungal (Ramachandra & Ravishankar, 2000), plant (Wu-Nan & Mutter, 1995), animal (Novais, 1998), or even isolated enzymes (Franssen & Walton, 1999). The use of microbial and fungal cell for the biotransformation has been extensively applied but the enzyme system of these sources are limited for certain types of chemical modifications (Hanson & Truneh, 1996; Ward & Singh, 2000), while the animal cells or the isolated enzyme are very much sensitive and it is difficult to handle them (Franssen & Walton, 1999).

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A cell suspension culture of plant origin has a vast biochemical potential for the production of specific secondary metabolites along with the ability to transform exogenous substrate into products of interest (Suga & Hirata, 1990). The plant cell cultures are relatively easy to handle as it has protective cell wall and have a diverse and efficient enzyme system as compared to other living organisms like animal cells, bacteria and fungi. Due to these advantages transformation of value added compounds using plant cells is gaining much attention (Huang & Ferguson, 2001).

In the current study, the effects of various factors such as illumination type and concentrations of plant growth regulators on the callus induction suitable for cell suspension and establishment of cell suspension culture were evaluated. In another series of experiments, the potential of established *Actinidia deliciosa* cell suspension culture for biotransformation of (-)-Ambrox was also studied. Since no sufficient data is available regarding the callus induction and the establishment of cell suspension culture of *Actinidia deliciosa* and its use in biotransformational studies, the results generated by these experiments are of much importance for the further use of this plant in the biotransformational studies.

Materials and Methods

Plant material and sterilization: Seeds were harvested from the mature fruit of *Actinidia deliciosa* plant and washed thoroughly with running tap water. The seeds were then sterilized using 20% v/v Sodium hypochlorite solution for 20 minutes. Few drops of Tween-20 were also added to the solution to reduce surface tension to ensure the contact of sterilant with the plant material. After 20 minutes, seeds were removed from the Sodium hypochlorite solution and washed six times with autoclaved distilled water to remove the traces of sterilizing agents under Laminar Flow Cabinet.

Seed germination: For seed germination, half strength basal MS medium (Murashige & Skoog, 1962) with 25 gm/L sugar and 6 gm/L agar for solidification was used. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving. Media sterilization was performed by autoclaving at standard temperature and pressure (15 PSI, 121°C, for 20 min.). The sterilized seeds were transferred to the germination medium and the cultures were kept under 16 hrs of illumination provided by White Fluorescent Light (1000 Lux) at 24±1°C for 8 weeks.

Induction of callus: Six weeks old *In vitro* grown plantlets of *Actinidia deliciosa* (Fig. 1A) were selected and leaves were used for the induction of calli. A series of experiment were conducted using full strength MS basal medium (Murashige & Skoog, 1962) 25 gm/L sugar supplemented with various concentrations of 2,4-D (2, 4-Dichlorophenoxy acetic acid) and IBA (Indole -3-butyric acid) in combination with BAP (6-Benzyl amino purine). The pH of the media was adjusted to 5.7 before autoclaving; agar was used as a solidifying agent at the concentration of 6 gm/L (w/v). Young leaves from the *In vitro* grown plantlets of 6 week old were harvested and small discs of 2mm were placed on the medium aseptically to initiate calli. The orientation of the leaf discs was abaxial in all experiments. After culturing, the jars were placed in the growth room either in complete darkness or under illumination, provided by white florescent lights of 1000 Lux, with 16 hrs photoperiod at 25±1ºC. The data were collected after one week to six weeks and callus induction rate with colour and texture were recorded (Table 1).

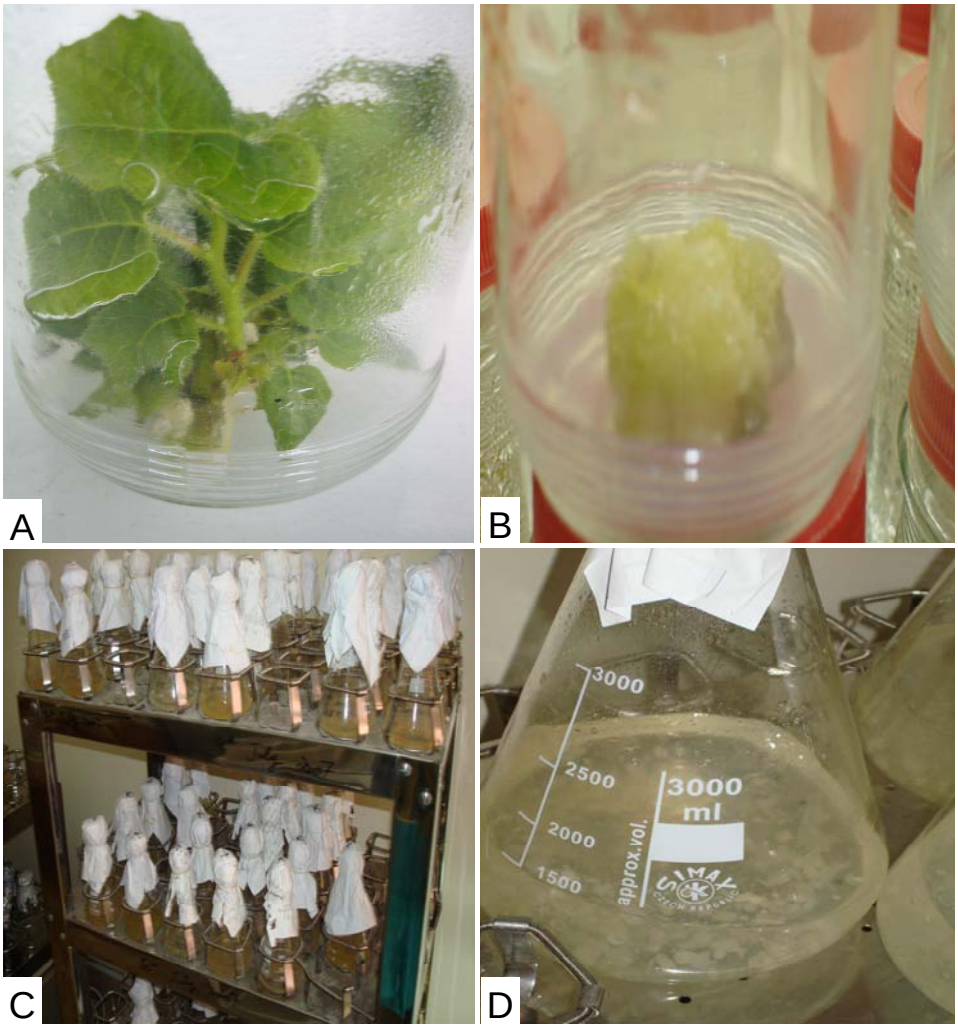


Fig. 1. A: *Invitro* grown Plant, B: Friable Calli, C: Preparation of Inoculum, D: Friable Cell suspension ready for biotransformation.

Establishment of cell suspension culture: The friable calli were chosen for further sub-culturing/ multiplication to establish cell suspension cultures and were subcultured on the same medium after every four weeks to maintain and multiply callus (Fig. 1B). For the establishment of cell suspension culture, the friable calli were transferred to 250ml Erlenmeyer flask containing 100ml of MS medium with the increased concentration of IBA i.e., 2.0 mg/L, without any solidifying agent (Fig. 1C). Two weeks old calli were used for the cell suspension culture. The flasks were placed on rotary shaker at a speed of 110 rpm for 15 days at 25ºC under a 16 hrs photoperiod. After 15 days, the cells were transferred to the bigger flask (500 ml) containing 200 ml of freshly prepared medium of the previous formulation (Fig. 1D).

Table 1. The effect of plant growth regulators on callus induction.

Media code	2,4-D mg/L	IBA (mg/L)	BAP (mg/L)	Percentage of Callus induction		Texture		Colour	
				Light (16 hrs)	Dark	Light (16 hrs)	Dark	Light (16 hrs)	Dark
I1	0.0	0.5	0.0	25	25	Watery	Compact	Green	Yellow
I2	0.0	1.0	0.0	50	25	Watery	Compact	Green	Yellow
I3	0.0	1.5	0.0	50	25	Compact	Compact	Green	Yellow
I4	0.0	2.0	0.0	75	50	Friable	Watery	Yellow	Yellow
IB1	0.0	0.5	0.5	25	25	Compact	Compact	Yellow	Whitish Yellow
IB2	**0.0**	**1.0**	**0.5**	**100**	**75**	**Friable**	**Compact**	**Green**	**Whitish Green**
IB3	0.0	1.5	0.5	50	50	Compact	Watery	Yellow	Yellow
IB4	0.0	2.0	0.5	25	25	Watery	Watery	Yellow	Yellow
D1	0.5	0.0	0.0	25	50	Compact	Friable	Yellow	Yellow
D2	1.0	0.0	0.0	75	50	Compact	Friable	Green	Yellow
D3	1.5	0.0	0.0	75	50	Compact	Watery	Green	Yellow
D4	2.0	0.0	0.0	75	75	Watery	Watery	Green	Yellow
DB1	0.5	0.0	0.5	25	25	Friable	Compact	Green	Yellow
DB2	1.0	0.0	0.5	100	75	Compact	Friable	Yellow	Yellow
DB3	1.5	0.0	0.5	50	50	Compact	Compact	Yellow	Yellow
DB4	2.0	0.0	0.5	50	50	Watery	Compact	Green	Yellow

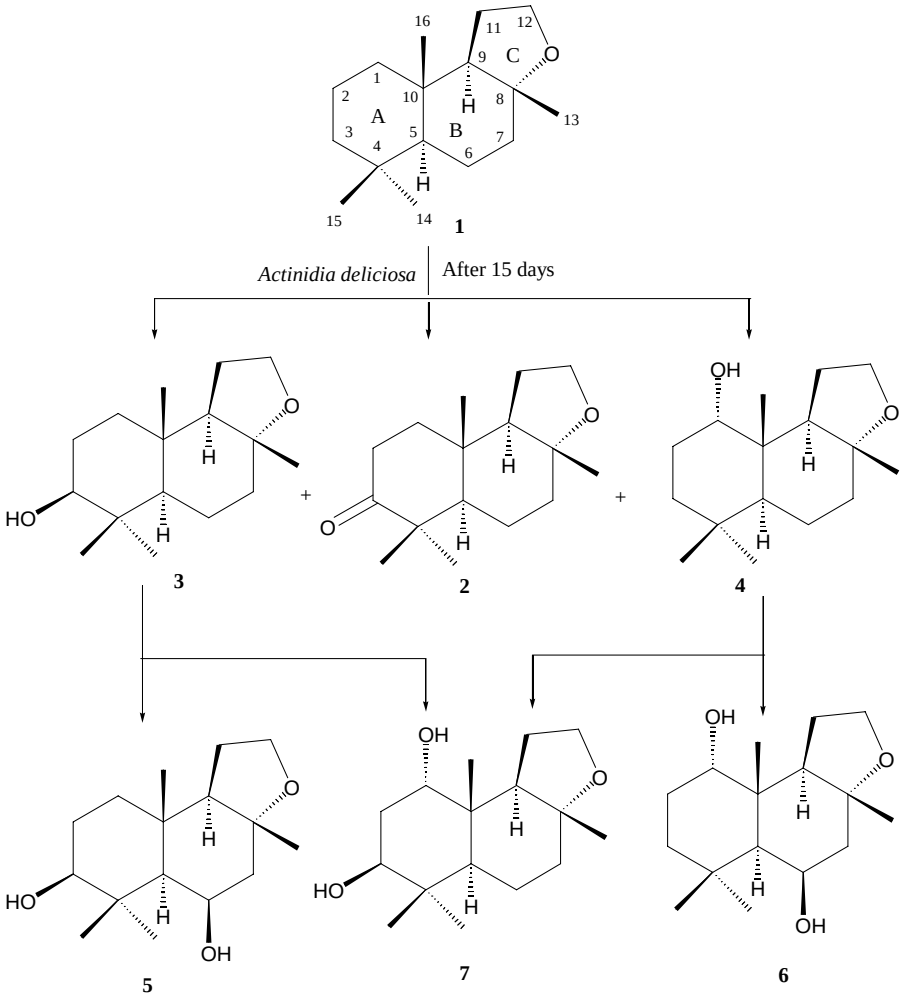
Biotransformation of (-)-ambrox: The established cell suspension culture of *Actinidia deliciosa* were used for the biotransformation of (-)-Ambrox as described by Nasib *et al*., (2006).

Results and Discussion

In this study, the primary objective was to identify the optimum conditions for the suitable growth of callus of *Actinidia deliciosa* plant. The establishment of cell suspension cultures for the biotransformational studies were also targeted. In this regard, along with the yield, the texture of the callus is of utmost importance since it ensures the equal dispersion of plant cells in the liquid medium. For the biotransformational studies, the callus should be friable which ultimately results in the formation of a very effective suspension culture. An efficient protocol was optimized for the callus induction in *Actinidia deliciosa*. The *In vitro* grown leaflets were used as an explant and the effects of illumination along with different concentrations of the plant growth regulators like IBA, 2,4-D and BAP on the callus induction and cell culture establishment was studied (Table 1).

All the explants, after one week of inoculation, showed some swelling on the medium containing IBA and BAP. After 30 days of culturing the medium containing 1.0 mg/L IBA with 0.5mg/L BAP (IB2), placed under 16 hrs of light (1000 Lux), showed remarkable results and almost 100% of the explant were converted into undifferentiated mass of cells. The colour and texture of that callus was light green and friable, respectively. The cultures on same medium which were placed in dark showed 75% calli initiation and it was of yellow colour with compact texture. However, medium containing 1.5 mg/L IBA with 0.5 mg/L BAP (IB3) only covered 50% of the explant with the callus in both dark and light conditions.

In the second series of experiments, 2,4-D was used with and without BAP. The medium contained 1.0 mg/L of 2,4-D with 0.5 mg/L of BAP (DB2) showed good results in terms of callus growth but the texture of the calli was compact, thus making it unsuitable to use for the establishment of suspension cultures.



Scheme 1. Biotransformation of Compound –(-)Ambrox by *Actinidia deliciosa* (Nasib *et al*., 2006).

Our results indicate that light have an important effect on the colour of the induced calli. The calli grown on IB2 medium were of green colour showed the presence of chlorophyll. The use of a BAP in combination with an auxin (either IBA or 2,4-D) highly influenced the texture of callus. As Table1 indicates, the medium formulations having only auxins either form a compact or a watery callus (high amount of intact water within the cells). The addition of BAP (a cytokinin) with an auxin increased the friability of the callus and ultimately ensures the formation of an efficient cell suspension culture.

Equal amount of the friable callus was added to the liquid medium. The liquid medium used for cell suspension culture were supplemented with 2.0 mg/L of IBA with 0.5 mg/L of BAP. The increment in the concentration of the hormone is due to the fact that high auxin to low cytokinin ratio increase the specific activity of enzymes which bring about the dissolution of the middle lamella of the plant cell walls and resulted in the dispersion of cells (George, 1993).

The callus was transferred on to the 250 ml flasks containing 100 ml of the liquid medium. The flasks were placed on gyratory shaker at 110 rpm. The media formulations stimulates the dispersion of callus in the liquid media and within 3-4 days of time the cells were evenly dispersed and clumps were dissociated. It is always necessary that a good suspension would result in the good result and a good suspension would only be possible if each and every cell is separated and have an equal chance to receive the exogenous substrate. During the incubation period the aliquots were analyzed for the transformation and conversion of substrate into negligible quantity (Fig. 1C). The reported media was found very suitable for the establishment of cell suspension culture of *Actinidia deliciosa* and the efficiency of the suspension was further proved by the biotransformational studies of the (-)-Ambrox (**1**), (Nasib *et al*., 2006). Since the compound (-)-Ambrox (**1**) was used earlier in bacteria and fungus to study the biotransformational activity of the enzymes, but when used in plant cells suspensions of *Actinidia deliciosa*, it afforded two new compounds 1α, 6β-dihydroxyambrox (**6**) and 1α,3β-dihydroxyambrox (**7**) besides 4 known compound 3-oxoambrox (**2**), 3β-hydroxyambrox (**3**), 1α-hydroxyambrox (**4**), 3β,6β-dihydroxyambrox (**5**) (Scheme-1) which were also isolated from fungus (Hanson & Truneh, 1996; Farooq & Tahara, 2000; Hashimoto *et al.*, 2001; Choudhary *et al*., 2004), hence the presence of two new compounds along with 4 known compounds proves the high and complex types of enzyme system to convert/utilize the substrate (-)-Ambrox (**1**) (Nasib *et al*., 2006). The structure of these compounds were deduced through spectral techniques and the structure of 1α, 6β-dihydroxyambrox (**6**) were characterized by X-ray crystallography discussed in detail Nasib *et al*., (2006).

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