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VARIOUS METABOLIC CHANGES DURING DIFFERENTIATION IN CALLUS CULTURE OF *Chlorophytum borivilianum*

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ABSTRACT

Chlorophytum borivilianum is an important medicinal plant and its tuberous roots are used for various health treatments. The plant regeneration protocol in *in-vitro* conditions have been developed for this medicinal plant. Best callus was induced when flower buds were cultured on MS medium supplemented with 1.0 mg/L BAP + 1.0 mg/L NAA. The above callus was subcultured on MS medium + BAP 1.0 mg/L + NAA 0.5 mg/L for root differentiation and on MS medium + BAP 1.0 mg/L for shoot differentiation. Metabolic changes occurring during callus differentiation were studied to quantify metabolites. Metabolites like total soluble carbohydrates, free amino acids increase during root and shoot differentiation from calli while reducing sugar decreases.

Keywords: *In vitro*, regeneration, callus differentiation, metabolites, *Chlorophytum borivilianum*

Introduction

India is one of those countries in the world where medicinal plants found their use in the well-developed system of medicine and have been known to be a rich repository of medicinal plants since ancient time. *Chlorophytum borivilianum* is an important medicinal plant distributed in India in hilly regions of Rajasthan, Madhya Pradesh, Maharashtra and Gujarat (Yadav *et al.*, 2004). Its tuberous roots are used in the treatment of rheumatism and used as revitalizer, as a remedy for diabetes, curative for natal and post-natal problems, also leucorrhea etc. (Thakur *et al.*, 2009).

Excessive collection from its natural habitat and destructive harvesting techniques coupled with poor seed germination and low vegetative multiplication ratio have made this

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species endangered (Narasimhan and Ravuru, 2003). Plant tissue culture is the only method for rapid clonal multiplication. *In vitro* propagation has been successfully employed for the conservation of medicinal crop genetic resources particularly those crops, which are vegetatively propagated (Agarwal *et al.*, 2004 and Indrayan *et al.*, 2004). Since to date, there have been few reports on micropropagation of *C. borivilianum* (Lattoo *et al.*, 2006).

Differentiation is a pre-requisite for application of biotechnology for crop improvement. Differentiation through callus culture involves changes in some of the biochemicals (Kumar *et al.*, 2010). Analysis of various cellular metabolites and enzyme activities provides a reasonable and promising approach towards an understanding of the biochemical basis of the developmental pathway (Singh *et al.*, 2009). This study was, therefore, undertaken to find out changes in the level of metabolites during root and shoot differentiation from callus cultures of *C. borivilianum*.

Materials and Methods

Flower buds and leaf segments from healthy plants are used as explants. MS basal medium fortified with 3% sucrose, myoinositol, pyridoxine HCl, glycine along with different concentrations of auxins and cytokinins were used individually and in combination for initiation and establishment of culture.

The pH of the medium was adjusted to 5.8 prior to addition of agar and autoclaving at 121^{o}C, 1.2 kg cm^{-2} pressure for 15 minutes.

A. Callusing

The explants were washed with detergent under tap water to remove dust particles. These were then surface sterilized with 70% alcohol followed by 0.1% mercuric chloride ($HgCl_2$) for 3-5 minutes and subsequently washed with double distilled water. The calli at first formed at excised edges of explants and then expanded throughout the explant. Juan *et al.* observed the highest frequency of callus formation from leaves in medium containing 0.5 mg/L NAA and 5.0 mg/L BAP in the case of *Catalpa bengei*. In the present investigation, callus growth was observed on BAP (1.0 mg/L) in combination with NAA (1.0 mg/L). Flower buds placed vertically in medium gave good callus induction than those placed in horizontal position.

B. Differentiation

After four-five weeks, the developed calli from flower bud were separated from explants and transferred to fresh medium of MS + 1.0 mg/L BAP + 1.0 mg/L NAA, thus using it as callus proliferation medium. For studies of metabolic changes during shoot and root differentiation, the calli were further sub-cultured for root differentiation on MS medium + BAP 1.0 mg/L + NAA 0.5 mg/L and for shoot differentiation on MS medium + BAP 2.0 mg/L.

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A number of various metabolites viz. total soluble carbohydrates, reducing sugar and free amino acids were studied on 2nd, 4th, 6th and 8th day on rooting medium and 0, 4, 8, 12 and 15th day after inoculation on shooting medium.

C. Extraction of metabolites

Extraction of metabolites was done by using supernatant of the sample.

For each sample, 100 mg of callus was homogenized in 80% ethanol and then homogenate was refluxed for 15 minutes on a water batch at 55°C and centrifuged for 15 minutes at 6000 rpm. This process was done three times and collected supernatant each time. The supernatant was pooled together and volume was made 10 ml. Then total soluble carbohydrates, reducing sugars and free amino acids were estimated.

These various metabolites were assayed during root differentiation as well as shoot differentiation at various days after inoculation of callus. These metabolites were also assayed in mature natural root tubers of *C. borivilianum.*

The root initiation was observed between 3-4th day followed by complete rooting on 6th day. But shoot initiation was observed 10th-14th day before which green patches formation occurred from 8th-10th on calli. The undifferentiated callus was served as control.

Results and Discussion

The basal medium without growth regulators failed to response for callus growth from the flower bud. Varying concentration of growth hormones to MS basal medium resulted in callus induction but frequency varied with the concentration of growth hormones.

MS basal medium supplemented with BAP (1.0 mg/L) and NAA (1.0 mg/L) showed the maximum percentage of callus induction (90.8%). A similar result was reported by Tilkat and Onay (2009) on P. vera including *P. atlantica* sub-species. Yang *et al.* (2006) produced compact greenish callus in *Acacia crassicorpa* on MS medium containing NAA.

The above callus was subcultured for shoot differentiation on MS + BAP (2.0 mg/L) while for root on MS + BAP (1.0 mg/L) + NAA (0.5 mg/L) and both roots and shoots on MS + BAP (1.0 mg/L) + NAA 0.01 mg/L. The role of auxins and cytokinins in callus induction was also advised by Kumar and Singh (2009) in *Stevia* rebaudiana, Goel and Singh (2009) in *Peganum harmala*, Lal and Singh (2010) in *Celastrus Paniculatus*.

The callus tissue is studied for metabolite estimation.

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D. Total soluble carbohydrates

Total soluble carbohydrates concentration increases in callus upto roots and shoots differentiation and after that declined showing high carbohydrate requirement to induce the root and shoot formation in callus (Yadav *et al.*, 1995).

A steady rise in total soluble carbohydrates was observed from 2nd day onwards in root differentiating calli till the initiation of roots i.e. 6th day and after that decrease was observed but not less than control (Fig. 1).

In the case of shoot differentiating calli carbohydrate content increases upto 12th day but declined on 15th day. But overall result showed that differentiated calli had more soluble carbohydrate than undifferentiated calli.

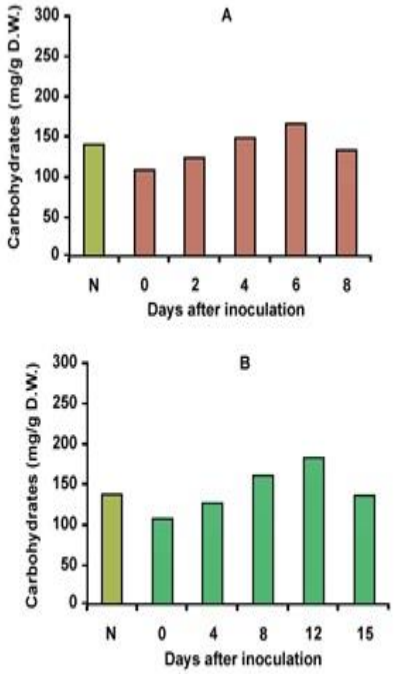

Fig.1: Histogram representing total soluble carbohydrates in *C. borivilianum* natural roots (N) and callus prior to inoculation (0 day) on rooting medium (A) at BAP 1.0 mg/l + NAA 0.5 mgl and on shooting medium (B) at BAP 2.0 mg/l after inoculation

E. Reducing sugars

The reducing sugar content declined gradually during shoot differentiation but in roots, differentiation content declined first four days and later on increases during root formation. In leaf callus of Simmondsia reducing sugar were high in callus proliferation medium which further increases significantly in shoot differentiating cultures (Kumar *et al.*, 2009).

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Natural root tubers contain very less reducing sugar. In root differentiating calli the decline in content was observed before root initiation i.e. upto 4th day but content increases during root initiation and decrease at the time of complete rooting. In shoot differentiating calli steady decrease in the content of reducing sugar was observed upto shoot formation i.e. on 15th day (Fig. 2).

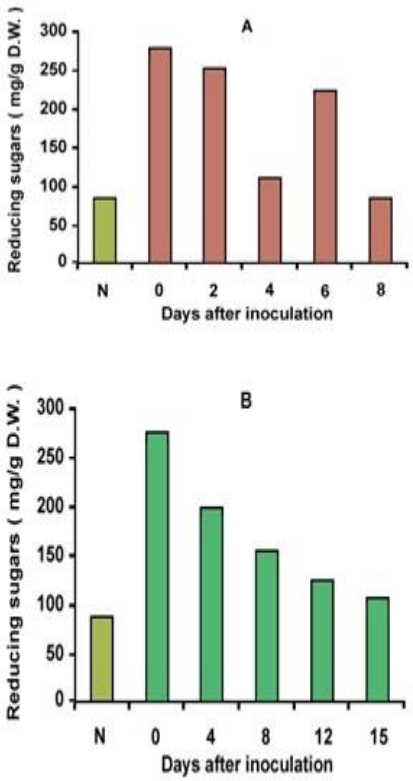

Fig.2: Histogram representing reducing sugar content in *C. borivilianum* natural roots (N) and callus prior to inoculation (0 day) on rooting medium (A) at BAP 1.0 mg/l + NAA0.5 mgl and on shooting medium (B) at BAP 2.0 mg/l after inoculation

F. Free amino acids

The free amino acids were also generally increased in callus during root and shoot differentiation and after that decline. The free amino acids in natural root tubers were low. During root differentiation from callus, free amino acids content increases upto 4th day after inoculation i.e. on root initiation. During shoot differentiation from callus, free amino acids content increases upto 12th day and decreases on 15th day (Fig. 3).

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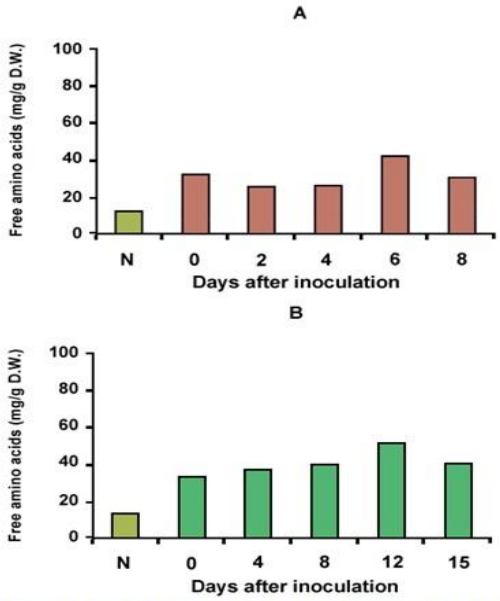

Fig.3: Histogram representing free amino acids in *C. borivilianum* natural roots (N) and callus prior to inoculation (0 day) on rooting medium (A) at BAP 1.0 mg/l + NAA 0.5 mgl and on shooting medium (B) at BAP 2.0 mg/l after inoculation

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