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Biochemical Changes During Rhizogenesis in Callus Cultures of *Tribulus Terrestris* Linn

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ABSTRACT

Biochemical changes during *in vitro* root formation in *Tribulus terrestris* were investigated in the callus derived from nodal explants on Murashige and Skoog's (MS) medium supplemented with 1.0 mg/l 2,4-D+ 0.5 mg/l Kinetin. The callus maintained on MS medium fortified with 2.0 mg/l 2, 4-dichlorophenoxy acetic acid (2,4-D) + 0.5 mg/l Kinetin. The above callus subcultured on Murashige and Skoog's (MS) medium + 2.0 mg/l α-naphthalene acetic acid (NAA). Metabolites like starch and total; soluble sugars decreased while reducing sugar, total phenol and total protein increased during root differentiation in callus. Enzyme activity viz. α-amylase, acid invertase, peroxidase and acid phosphatase increased during root differentiation.

Keywords: *Tribulus terrestris* Linn, Biochemical changes, Rhizogenesis, Callus.

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INTRODUCTION

Tribulus terrestris Linn. belongs to the family Zygophyllaceae, is a trailing plant common in sandy soil, has been described to be of great medicinal value. It is a reputed drug in Ayurvedic system. This plant is commonly known in Hindi: Chota gokhru. In India, *T. terrestris* fruits have long been used as a tonic, a diuretic against kidney diseases and stones and for treating impotence[1]. This plant species is conventionally propagated through seeds but the germination percentage is very low under natural and laboratory conditions[2]. So tissue culture is the rapid process for the mass propagation of this plant because this species is prone to extinction due to habitat destruction and overexploitation. The process of growth and morphophysiological specialization of cells from unorganized mass of callus cells i.e. differentiation, is a prerequisite for the application of biotechnology for conservation of medicinal plants. Differentiation of organized structure is controlled by growth regulators such as auxins and cytokinins along with other components of the culture media. Differentiation through callus cultures involves changes in some of the metabolites[3,4]. But little is known about the biochemicals events occurring in the cultures cells undergoing rhizogenesis. 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. The present study was, therefore, undertaken to determine the changes in the levels of metabolites and enzymes during root differentiation from callus cultures of *T. terrestris*.

MATERIALS AND METHOD

Nodal explants were excised from the young flowering twig of *Tribulus terrestris*. These were then surface sterilized by washing with teepol solution (5% v/v) for 10 min followed by washing with running tap water. These then further surface sterilized with 70% ethanol (1 min) followed by 0.1% mercuric chloride (3 min) and finally washed 4-5 times with sterile double distilled water. The surface sterilized explants were inoculated on MS medium[5] containing 3% sucrose and 0.8% agar (w/v) along with growth regulators 1.0 mg/l 2,4-D and 0.5 mg/l Kn. The pH of the medium was adjusted to 5.8 using 1NNaOH or 1N HCl solution before being autoclaved at 121^{o}C, for 15 min. The sterilized explants were cultured under laminar air flow. The nodal callus was maintained on MS medium supplemented with 2.0 mg/l 2,4-D mg/l and 0.5 mg/l Kn. The callus was subcultured on root forming medium MS+ NAA (2.0 mg/l). Sampling was done prior to inoculation (0 day) and on 4th, 8th, 12th and 16th day of inoculation on root differentiation medium.

All the cultures were incubated at 25±2^{0}C under a photoperiod of 16 h light and 8 h of darkness by cool white fluorescent light.

Extraction and determination of metabolites

Extraction of metabolites was done by the modified method of Barnett and Naylor [6]. One hundred mg of dry callus was homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10,000 g. The extraction procedure was repeated three times with the residue. The supernatants were pooled and the final volume was made to 5 ml with ethanol and the total soluble sugars[7], reducing sugars[8], total phenols[9] were estimated. The pellet was hydrolyzed with 4 ml of chilled 0.2 N $HClO_4$ at 40C for 24 h. The hydrolysate was centrifuged at 5000 g for 15 min and the supernatant was used for starch estimation. Total soluble protein from the callus was extracted in 0.1 M Tris-HCl (pH 7.5) by using pre-chilled pestle and mortar. The homogenate obtained was centrifuged at 10,000 g for 15 min at 4^{0}C and total soluble proteins in the supernatant were estimated using the method of[10].

RESULTS AND DISCUSSION

The nodal explants showed callogenesis on MS medium supplemented with various concentrations of 2, 4-D and Kinetin. However, all of the explants showed early callus initiation on MS medium supplemented with 2,4-D (1.0 mg/l) + Kn (0.5 mg/l), indicating that explants require an optimum concentrations of growth regulators for differentiation (Table 1 and Figure 3 A and B). The effectiveness of 2, 4-D and Kn for callus induction has been also reported in many plants viz. *Peganum harmala*[11], *Cardiospermum halicacabum*[12] and *Withania somnifera*[4]. The subculturing of callus after 4 weeks on MS medium supplemented with2, 4-D (2.0 mg/l)+Kn (0.5 mg/l) showed good growth of callus. The above callus when subcultured on MS medium without growth regulators failed to show growth as well as organogenesis. When various concentrations of different plant growth regulators (Table 1) were supplemented to MS medium, roots appeared after 14-34 days of inoculation with poor to good growth callus. Out of these combinations, NAA (2.0 mg/l) was found to be best for growth of callus as well as for early root induction. Also, callus showed good growth on combinations Kn (0.2 mg/l)+IBA (0.1 mg/l) but initiation of root was observed after 30 days of inoculation. Thence, for root differentiation, callus was subcultured on root differentiating medium MS + NAA (2.0 mg/l). The visible appearance of root was observed after 14 days of subculturing (Figure 3). This suggested that differentiation in *T. terrestris* is dependent on the concentration of auxins in the medium. Similar results have also been reported in *Chlorophytum borivilinum*[13], *Withania somnifera*[14]. Starch content slightly increased after 4th day of inoculation as compared to control calli. Thereafter, it decreased and decline was observed up to

12th day i.e. root initiation, though a little increase was observed after the formation of roots on 16th day. Reducing sugars content increased after inoculation of callus on root forming medium. However decreased at 8th day and slight increase was observed `after root initiation (12th day). The content of reducing sugar decreased after visible appearance of roots. On the other hand the α-amylase activity slightly decreased up to 4th day and then marked increase in the activity was observed followed by sharp decrease on 16th day. Higher α-amylase activity indicates the starch degradation, as differentiation is a high energy requiring process, therefore, high α-amylase activity necessary for mobilization of carbohydrate reserves. The decreased in reducing sugar content before and after root appearance has been suggested to be associated with the utilization of sugar during the differentiation[15,16,17]. Total soluble sugars was less in control than differentiated calli but the calli with root appearance i.e. after 16th day had less total soluble sugars content than control one. A little decrease in content was observed during root appearance from calli. However, the acid invertase was more in undifferentiated callus (control) and consistently decrease 4th to 8th day. The enzymic activity increased at the time of root initiation, however, it decreased after root emergence on the 16th day. As the acid invertase activity is related to sucrose uptake and removal of sugar from the vacuole, the decrease in reducing sugar content and acid invertase activity has direct correlation with differentiation as suggested earlier also[18,19]. Total proteins were higher in root differentiated calli as compared to control. The content increased sharply at 4th day, however, more or less same content (though decreased) was observed from 8 to 16th day i.e. before and after the root formation. The content of total protein decreased during differentiation and increased later on was also observed in *Cardiospermum halicacabum*[20,16]. Total phenolic content was less in differentiated calli as compared to controlled callus. The quantity of phenolic content decreased up to 8th day, but the root formation was accompanied by a increase in total phenol content i.e. 12th day, however, decrease in phenol content was observed during root emergence on 16th day. On the other hand peroxidase activity was high in differentiated calli than control calli. The activity of peroxidase increased on 4th day and little increased was observed up to 12th day, however decreased during root emergence i.e. 16th day. There was a gradual increase before and after differentiation. The increased content of phenol during differentiation indicates their involvement in organ formation as they are known to facilitate oxidation of IAA and lignifications of cells by cross linking of cell wall constituents via peroxidase catalyzed oxidative polymerization of hydroxilated cinnamyl alcohols[21]. Since the final phase of in vitro root differentiation involves the deposition of lignin[22], the increase in acid peroxidase activity during root differentiation in present study could be related to continuous root formation. Similar findings had also been reported[23].

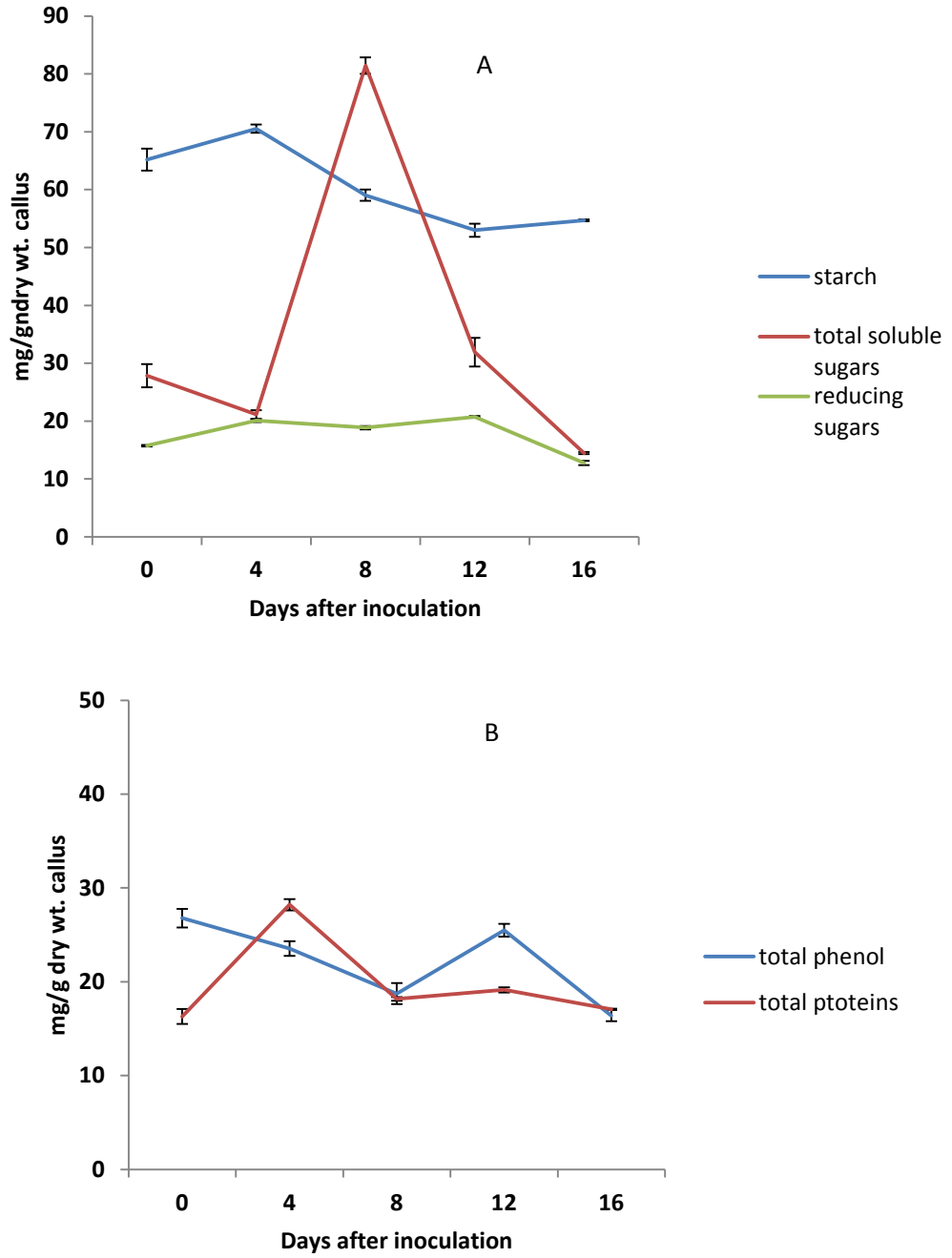
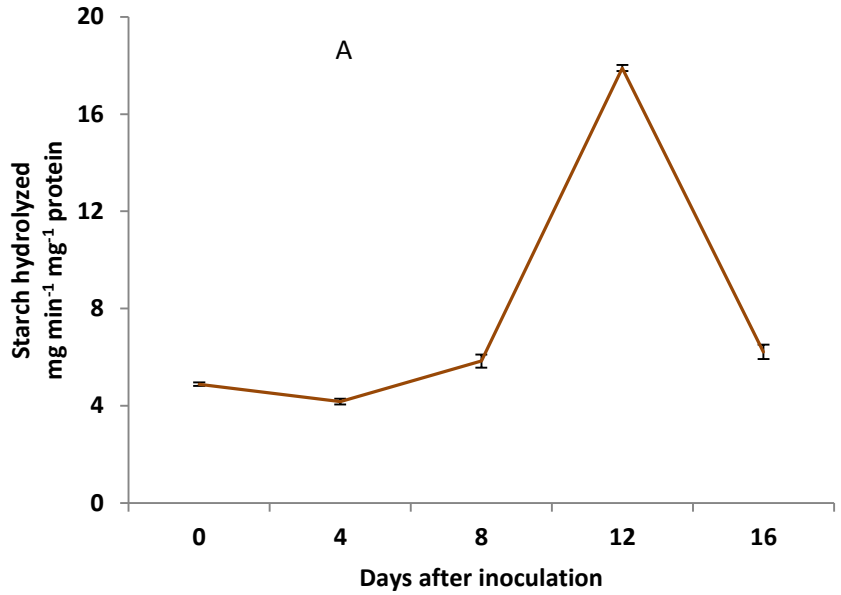
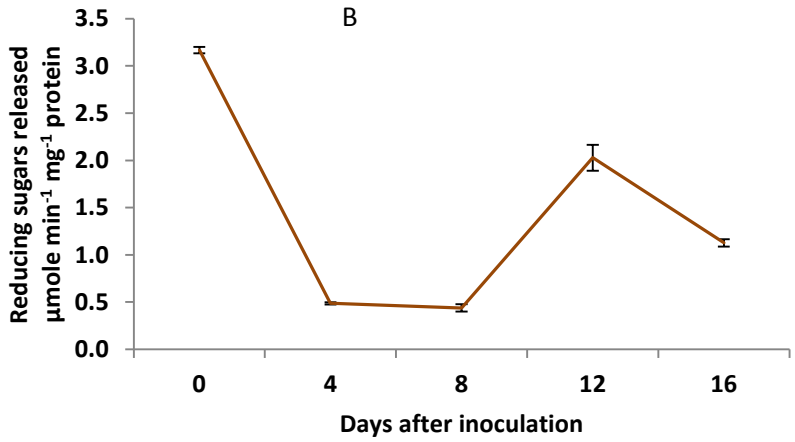
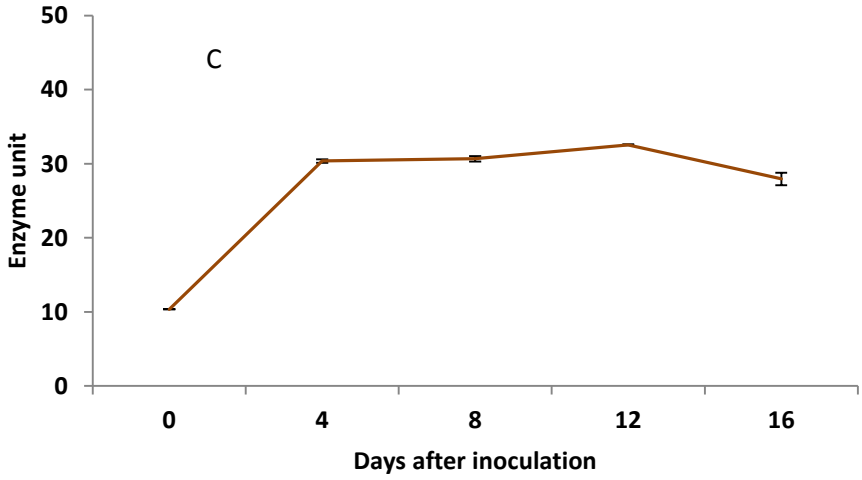


Figure 1: Changes in the levels of (A)- starch, total soluble sugar and reducing sugars; and (B)- total phenol and total proteins in *T. terrestris* callus prior to inoculation on root differentiating medium (0 day) and on 4th , 8th , 12th and 16th day of inoculation (Bars indicate ± SE).


A
Starch hydrolyzed
mg min-1 mg-1 protein
20
16
12
8
4
0
0
4
8
12
16
Days after inoculation


B
Reducing sugars released
µmole min-1 mg-1 protein
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
Days after inoculation


C
Enzyme unit
50
40
30
20
10
0
0
4
8
12
16
Days after inoculation

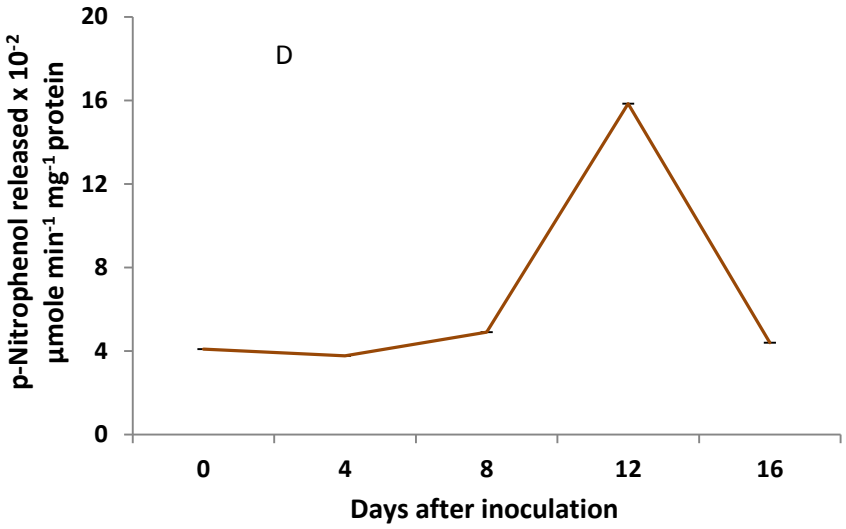


Figure 2: Changes in the levels of (A)- α-amylase (B)- acid invertase (C)-peroxidase and (D)-acid phosphatase activity in *T. terrestris* callus prior to inoculation on root differentiating medium (0 day) and on 4th , 8th , 12th and 16th day of inoculation (Bars indicate ± SE).

Table1: Effect of growth regulators supplemented to MS medium on callus of nodal explant for root differentiation (observations recorded upto 6-weeks after inoculation)

Medium + growth regulator (mg/l)	Visual growth of callus after 6-weeks *	Colour and Texture of callus	Differentiation (Days required)
Control	—	--	--
NAA (1.0)	++	Whitish, non-friable	Roots (27)
(2.0)	+++	Golden, non-friable	Roots (14)
(3.0)	+	Golden, non-friable	Roots (29)
(4.0)	+	Creamish, non-friable	--
IBA (0.1)	++	Creamish, friable	--
(0.2)	+	Creamish, friable	Roots (15)
(0.5)	+	Creamish, friable	--
BAP (1.0)			
+ NAA (0.5)	++	Creamish, friable	Roots (19)
(1.5)	+	Creamish, friable	--
(2.5)	+	Creamish, friable	--
BAP (3.0)			
+NAA (1.5)	+++	Creamish, non-friable	Shoots (10)
(2.5)	++	Creamish, friable	Shoots (30), Root (34)
(3.5)	++	Green, friable	Shoot (32), Roots (25)
BAP (1.0)			
+Kn (0.1)	+	Creamish, friable	Roots (22)
(0.5)	++	Whitish, friable	Roots (19)
(1.0)	+	Whitish, friable	--
Kn (0.2)			
+IBA (0.1)	+++	Creamish, non-friable	Roots (30)

(0.2)	+	Creamish, non-friable	--
(0.5)	+	Creamish, non-friable	--

*– No callus, + Poor callus, ++ Moderate callus, +++ Good callus

Abbreviations: Murashige and Skoog's (MS), 2, 4-dichlorophenoxy acetic acid (2, 4-D), α-indole 3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 6-benzyl amino purine (BAP) and kinetin (Kn)

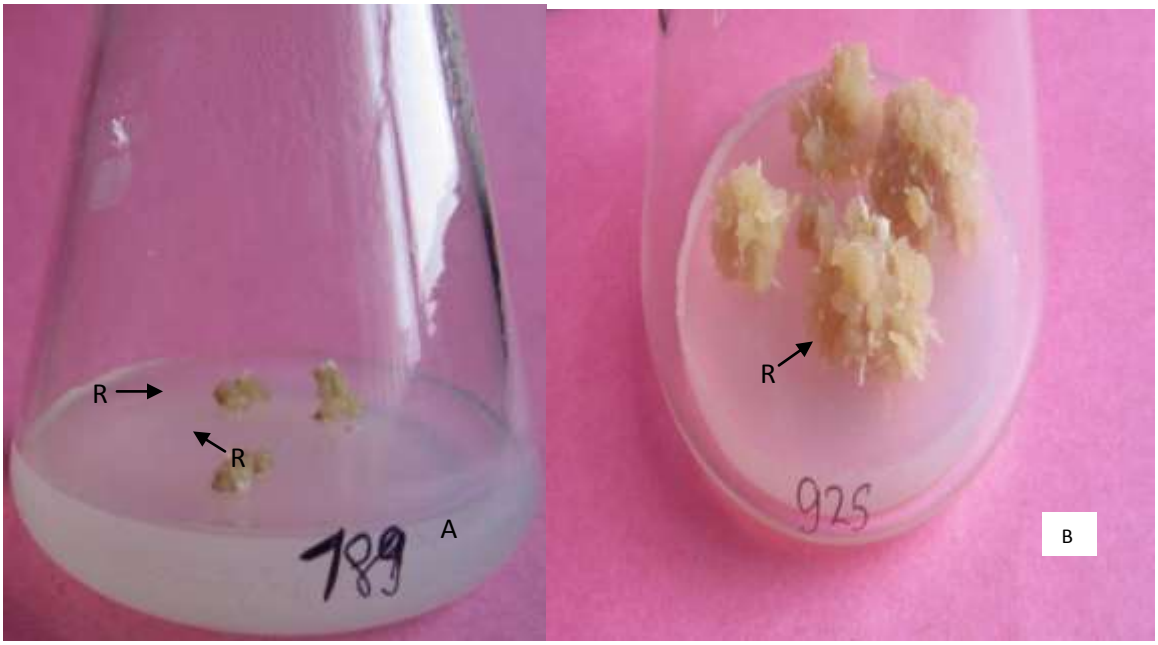


Figure 3: Differentiation of root on MS medium supplemented with (A) NAA (2.0 mg/l) and (B) Kn (0.2 mg /l) + IBA (0.1 mg/l) from callus of node explants (R-Root)

CONCLUSION

T. terrestris fruits is used as a tonic, a diuretic against kidney diseases and stones and for treating impotence. Germination percentage of *Tribulus* is very low under natural and laboratory conditions, so tissue culture is the advantageous process for the mass propagation of this plant because this species is prone to extinction. In the study, various metabolites like starch and total; soluble sugars decreased while reducing sugar, total phenol and total protein increased during root differentiation in callus. Enzyme activity viz. α-amylase, acid invertase, peroxidase and acid phosphatase increased during root differentiation

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