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PHENYL ALANINE AMMONIA LYASE (PAL) ACTIVITY AND ARTEMISININ CONTENTS OF *ARTEMISIA ANNUA* L., DURING CALLUS GROWTH

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ABSTRACT
The relationship between the level of phenyl alanine ammonia lyase and the concentration of artemisinin produced in the callus cultures of *Artemisia annua*. L was studied. TLC and high performance liquid chromatographic analysis indicated the presence of maximum artemisinin production was associated with the higher level of PAL activity at the 4th week of callus growth. Both artemisinin and level of enzyme activity declined subsequently by 5th week. Protein and nucleic acid contents of the callus during the growth showed an inverse relationship with the level of phenyl alanine ammonia lyase. The enzyme appears to play a cardinal role in the production of artemisinin during the callus growth.

INTRODUCTION

Artemisia annua. L belonging to the family Asteraceae produces an antimalarial drug artemisinin, which is effective against both Chloroquine resistant and Chloroquinine sensitive strains of Plasmodium falciparum, as well as against cerebral malaria. Synthesis of artemisinin has not proved to be commercially feasible and low yields are obtained from natural sources. Global scale production of artemisinin is not satisfactory consistent with the demand. There fore, an attempt to produce this secondary metabolite through tissue culture may be highly promising.

Artemisinin, a sesquiterpene lactone contains an endoperoxide bridge, has been isolated from aerial parts of *Artemisia annua*. L. plants. It is effective against both drug resistant and cerebral malaria causing strain of plasmodium falciparum. The relatively low yield (0.01-0.8%) of artemisinin in *A.annua* is a serious limitation to the commercialization of the drug.

PAL enzyme is one of the most intensively studied in plant secondary metabolism (Hrazdina, 1992; Lewis and others, 1999). Phenylpropanoid metabolism is related to the plant defense system and early studies in *Phaseolus vulgaris* had reported an increase in PAL activity and concentration of total phenols (Bolwell and others, 1985). PAL is considered to be a key regulatory enzyme for flavonoid/anthocyanin biosynthesis (Martinez and others, 1996). Flavonoids are synthesized by the phenylpropanoid pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl CoA.

Rapidly growing cells do not produce much, if any, secondary metabolite (Berlin *et al.,* 1985). Minimal growth rate favours secondary metabolite production (Yeoman *et al.,* 1980, 1982; Rokem and Goldberg, 1985). In this article an emphasis is made on the production of secondary metabolite, (artemisinin) its regulation by phenyl alanine ammonia lyase.

MATERIALS AND METHODS

Seeds were obtained from Orto Botanico Dell Universita, PADOVA, and Italy. The seeds harvested in the previous year crop were used as a source for raising callus cultures, since rapid loss of viability has been observed in the older seeds.

Seeds were surface sterilized with detergent for 3 min and 70% alcohol for 2 min followed by 0.05% mercuric chloride for 5 min. They were rinsed thoroughly thrice with sterile distilled water. The seeds were placed on 1/4th strength of Murashige and Skoog (1962) medium without vitamins and growth hormones with 2% sucrose and 0.8% agar to raise aseptic seedling. Ten-day-old whole aseptic seedling explants were transferred to MS agar medium supplemented with biotin (1mg/L) + myoinositol (100mg/L) and different plant growth hormones. The pH of the medium was adjusted to 5.7-5.8. The cultures were grown under continuous fluorescent light (PAR = 45μEm^2 s^{-1} at 25 $\pm$ 20ºC.)

Callus cultures was initiated from seedling explants of *A.annua* on MS (Murashige and Skoog, 1962) medium supplemented

with 1mg/L NAA-(1mg/L), Kn-(0.1mg/L), 3% sucrose and 1% agar (callus maintenance medium) by regular subcultures for every 2-3 weeks. Myoinositol (100mgL^{-1}), biotin (1mgL^{-1}) were used as adjuants. Cultures were grown at 25 ± 2°C under continuous darkness.

Extraction of artemisinin

2.5g of 3 week old callus tissue was taken for the analysis of artemisinin were homogenized in a mortar with sand using petroleum ether (b.p.60°-80°C) and the contents were extracted using soxhlet apparatus. The extracts were filtered, concentrated on a rotary evaporator. The residue was dissolved in acetonitrile: water (1:1) was used for HPLC analysis.

TLC was carried out over silica gel 'G' plates using hexane and ethyl acetate as solvent. The spots were visualized by keeping the plates in a container with iodine vapours.

HPLC for quantification of artemisinin

Sample separation

400μL of acetonitrile-water (1:1) mixture containing artemisinin was filtered through a membrane and the clear supernatant was used for HPLC analysis. 10 μL sample was injected to HPLC.

Authentic samples

One mg of standard artemisinin obtained from Sigma Chemical Co was dissolved in 400 μL of acetonitrile-water (1:1) and 10 μL was injected to HPLC.

Column conditions

Column	:	U-Bonda-Pack C^{18} column
Detection	:	UV at 216 nm
Mobile phase	:	Acetonitrile-water (1:1)
Flow rate	:	1mL/min
Temperature	:	Ambient

Extraction and estimation of phenyl alanine ammonia lyase

Extraction

500mg of callus tissue was homogenized in a mortar, using 0.1M borate buffer (pH 8.8), 250mg of activated charcoal and acid washed sand. The extract was filtered and centrifuged at 14,000 rpm (30,000xg) for 15 min. The above steps were carried out at 3°C. The clear supernatant containing the enzyme was taken and the volume was made up to 10 mL with buffer and used for the assay.

The reaction mixture contained 0.5 mL of enzyme extract, 1.0mL of L-Phenylalanine (0:06 M) and 1.5mL of 0.1 m borate buffer (pH 8.8) in a total volume of 3mL. Blank contained 0.5ml of buffer instead of enzyme extract.

The reaction mixtures were incubated at 30°C, in a controlled water-bath, for one hour and the amount of cinnamic acid formed was measured at 290nm, using UV visible spectrophotometer. The amount of cinnamic acid formed was calculated, using the standard graph prepared, by taking different concentrations of cinnamic acid in borate buffer. One unit of enzyme activity was expressed as gram of cinnamic acid formed per mg protein per hour.

RESULTS AND DISCUSSION

Growth curve of callus cultures

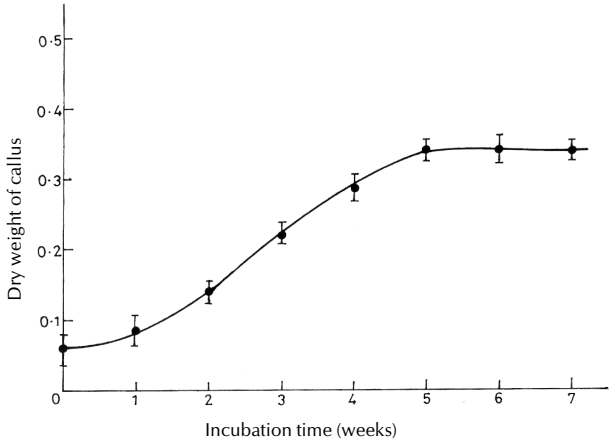

Figure 1: Growth curve of callus cultures of *A. annua*

Growth profile of the callus cultures of *A.annua* grown on NAA and Kn medium showed sigmoidal curve (Fig.1). The callus showed a lag phase of 2 weeks followed by an exponential phase of 5 weeks followed by stationary phase, starting from the 5th week. Maximum growth was obtained on the 5th week of culture with increase in callus on dry weight basis. The increase of growth was 7 fold over the initial inoculum (Fig. 1).

Growth and artemisinin content in callus cultures

The growth and artemisinin content of callus cultures maintained on MS medium with NAA (1mg/L), Kn (0.1 mg/L) and 3% sucrose as supplements were monitored over a period of 5 weeks. The growth pattern showed a typical sigmoidal curve with maximum growth at the 5th week of culture (Fig. 2). *A.annua* callus cultures showed a lag phase of 5 weeks, followed by an exponential phase increased by 4.8 fold over the initial inoculum during the growth cycle.

Artemisinin content was also studied throughout the culture period. Maximum production of artemisinin was observed on the 3rd week of culture which was about 2.4 fold increase on a fresh weight basis and maximum callus growth on the 5th week of culture during the growth cycle.

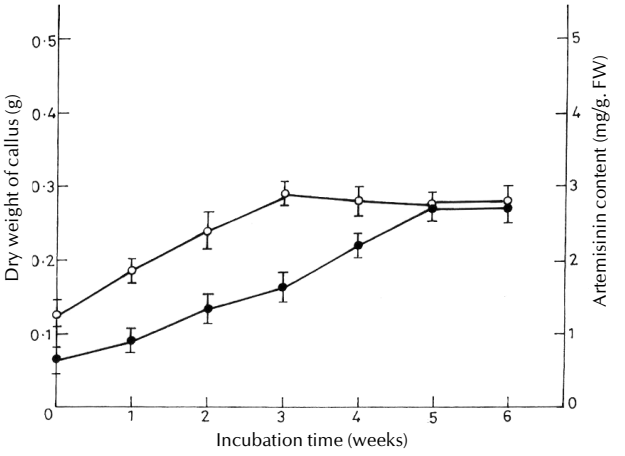

Figure 2: Growth and artemisinin content in callus cultures of *A. annua*

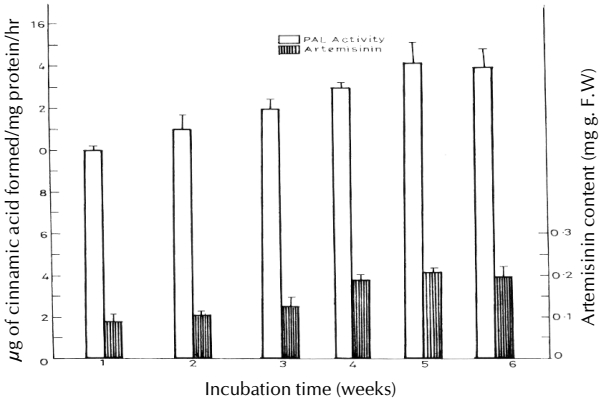

Figure 3: Changes in the PAL activity and artemisinin content of callus cultures of *A. annua*

PAL activity and artemisinin content

PAL activity of callus cultures and Artemisinin content was determined at weekly intervals for a period of 6 weeks. The level of PAL activity increased upto 5th week followed by a decline. Artemisinin content also increased during 5th week followed by a 2 decline (Fig. 3).

There was a close relationship between PAL activity and artemisinin content. Artemisinin content and PAL activity increased from first week to 6th week of culture and then declined (Fig.3). Decreased growth rate and increased PAL activity inturn increased accumulation of artemisinin (Fig.3). It was reported (Phillips and Henshew 1977) that in the cultures of *Sycamore,* the enhanced protein synthesis reduced the synthesis of phenolics.

Phenylalanine is a precursor of flavonoids and phenolics synthesis. It is assumed that biosynthesis of polyphenolic compounds may compete with protein synthesis for phenyl alanine and that secondary metabolites synthesis may be inhibited because of limiting availability under conditions of rapid incorporation into protein in the protein comprtition model. Strissel *et al.,* 2005 also observed down-regulated PAL activity and reduced flavonoids accumulation in young apple leaves supplied with high nitrogen fertilization.

Sakuta *et al*. (1986) proposed growth linked and non-growth linked hypothesis, correlating growth and secondary metabolite production in plant cells in culture. Accumulation of secondary metabolites in most of the systems studied, showed non-growth linked pattern or at the stationary phase (Yeoman *et al.,* 1980). This was shown in the production of rosemarinic acid from *Anchusa officinalis* (De Eknamkul and Ellis 1985) or alkaloid production in *Ailanthus altissima* (Anderson *et al.,* 1983). It was envisaged that in non-growth linked pattern of secondary metabolite production, late expression of the bio synthetic enzymes might lead to production of metabolites (Barz, 1977). Growth linked accumulation of secondary metabolites has been reported in production of betacyanin from *Phytolaca americana* (Sakuta *et al.,* 1986), nicotine from Nicotiana tobaccum (Ravishankar and Mehta, 1982), and betalains from *Chenopodium rubrum* (Berlin *et al.,* 1986).

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