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EFFECT OF GROWTH REGULATORS AND AMINO ACIDS ON ARTEMISININ PRODUCTION IN THE CALLUS OF *ARTEMISIA ABSINTHIUM*

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Abstract

Studies were conducted on the effect of amino acids and growth regulators on the production of artemisinin, an antimalarial compound, in the callus of *Artemisia absinthium*. Callus was initiated on solid MS medium supplemented with BAP and NAA from leaf explant. For the production of artemisinin, the callus was proliferated on sterile filter paper bridge in MS medium supplemented with different concentrations of plant growth regulators and amino acids. Estimation of artemisinin contents showed that leaves contained 223 µg/g artemisinin while no artemisinin was observed in the stem extract. Callus culture initiated from leaf explant on MS medium without any growth regulator, failed to show the presence of artemisinin. The amount of artemisinin in the callus culture was influenced with the addition of different growth regulators and amino acids to the medium; 3.1 µg/g artemisinin was present in the callus cultured on MS medium to which valine (12.5 mg/l) was added. Addition of cystine (12.5 mg/l) to the medium resulted in 2.8 µg/g artemisinin production. The amount of artemisinin in the callus cultures was 3.05µg/g and 1.95 µg/g when BAP (2 mg/l) and NAA (2 mg/l), respectively were present in the medium. Addition of other growth regulators and amino acids resulted in nominal or no artemisinin production. Present study suggest that artimisinin production can be enhanced with the manipulation of medium by different hormones and amino acids in the callus cultured on sterile filter paper bridge.

Introduction

The genus *Artemisia* consisting of about 400 species, probably is the largest and the most widely distributed genus of the Asteraceae. The essential oil obtained from *Artemisia absinthium* wild plant is mainly used as flavouring agent in food and beverages besides having antibacterial, antifedant, antipyretic, anti infertility, cytostatic and antimalarial activities (Kaul *et al*., 1976; Abivardi & Benzi, 1984; Khattak *et al*., 1985; Rao *et al*., 1988; Zafar *et al*., 1990). The plant has been used by the Chinese to treat patients infected with *Plasmodium vivax* and *P. falciparum*, resistant to quinine and its analogue (Schmidt & Hofheinz, 1983; Xu *et al*., 1986).

Although the chemical composition of *Artemisia absinthium* has not been fully characterized, a number of compounds including terpenes as limonene, myrcene, α and β thujone (Vostrowsky *et al*., 1981; Tucker & Maciarello, 1993), the sesqueterpene, caryophellene (Tucker & Maciarello, 1993) and sabinyl acetate and chrysanthenyl acetate (Chilava *et al*., 1983) have been identified. Artemisinin and its derivatives have a “reserve status” in the World Health Organization essential drugs list for using it in areas with multi-drug resistant malaria, such as Southeast Asia (Muller *et al*., 2000). Its complex structure makes chemical synthesis impractical, thus the production of the sesqueterpenes by tissue culture technique has become a viable possibility (Nam-cheol *et al*., 1992).

Concentration of artemisinin in different *Artemisia* species has been found to vary in different geographical environments. It may also vary in the same plant from time to time. Range of variability in artemisinin content in naturally occurring population of *Artemisia annua* is very low (0.01% to 0.15%) (Srivastava, 1999).

Plant cells are biosynthetically totiopotent (Rao & Ravishankar, 2002). Secondary products however are generally not produced in significant quantities until a culture reaches stationary phase (Lindsey, 1985). During the last decades many approaches have been used to improve the usually low content of secondary metabolites in cell cultures. The optimization of culture conditions has resulted in 10-20 fold increase in metabolites production (Deus-Neumann & Zenk, 1984).

Plant cell culture technology and its optimization provide a continuous and constant year round supply of natural plant products. Methods for the production of important active compounds have been developed in some *Artemisia* sp., such as *A. vulgaris* (Benthrope & Brown, 1989), *A. douglasina* (Pestchanker *et al*., 1989), *A. pallens* (Benjamin *et al*., 1990) and *A. annua* (Whipkey *et al*., 1992; Paniego & Giulietti, 1994).

Work has been done both with cell free systems with artemisinic acid as a precursor (Nair & Basile, 1992) and with cell culture systems. Extracts of these cultures have been assayed for antimalarial activity, and they have been found to be a potentially useful means of producing artemisinin (Nair *et al*., 1986; Liu *et al*., 1992). Although it is thought that the production of artemisinin is correlated with cell differentiation (Jaziri *et al*., 1995), Nin *et al*., (1997) reported that plant genotype and manipulation of culture environment and media can also affect the rate of both cell growth and accumulation of secondary compounds in *Artemisia absinthium*.

Growth regulator concentration is often a crucial factor in secondary product accumulation (DiCosmo & Towers, 1984; Deus-Neumann & Zenk, 1982). Mantell & Smith, 1984 reported that the type and concentration of auxins and cytokinins or the auxin/cytokinin ratio, dramatically alters both the growth and the product formation in culture plant cells. Under some stress-inducing conditions, the regulation of amino acids pathways may be dominated by the need for secondary metabolites derived from the pathway rather then protein synthesis (Niyogi & Fink, 1992). This study was conducted to examine the effect of precursor feeding (plant growth regulators and amino acids) on the production of artemisinin in the callus of *Artemisia absinthium*.

Materials and Methods

Leaf explants from *In vitro* grown seedling on plain agar medium (3% sucrose and 0.8% agar) were cultured on solid MS medium (Murasshige & Skoog, 1962) supplemented with 0.5 mg/l BAP (6-benzulamino purine) and 0.1 mg/l NAA (α-nephthalene acetic acid) for callus induction. After four weeks of callus induction, in order to find out the effect of growth regulators and amino acids on the production of artemisinin, 0.1g callus was placed on sterile filter bridge in the flasks with liquid MS medium supplemented with different concentrations of growth regulators and amino acids. Two concentration of plant growth regulators (1mg/l and 2mg/l) were used because high concentration inhibits callus proliferation. A 2.5mg/l and 6 times higher concentration (12.5mg/l) of amino acids were used in MS medium. The flasks were kept in a culture room with 16/8 hour day/night cycle and 25±1 ºC for callus proliferation. After three weeks morphology of callus was observed and artemisinin content was analyzed.

Extraction procedure: The extraction of artemisinin from callus was made following the procedure of Kim *et al*., 2001. Fresh callus (2 g) was homogenized in 5 ml toluene for 10 minutes at 10000 rpm followed by sonication for 15 minutes. The homogenate was centrifuged at 4500 rpm for 10 minutes at 4 ºC and supernatant was collected. The pellets were again extracted in 3 ml toluene and centrifuged. Both supernatants were mixed and dried in open air at 20 ºC and stored at -20 ºC until analysed using HPLC.

Endogenous level of artemisinin: Endogenous level of artemisinin was measured according to Smith *et al*., (1997). The dried extracts were solublized in mixture containing 100 µl methanol and 400 µl of 0.2% (w/v) NaOH. The mixture was hydrolyzed for 45 minutes at 50 ºC. The reaction was stopped by adding 400 µl of 0.2 M acetic acid and test tubes were placed in ice cold water. The final volume 1.0 ml was made by adding 100 µl methanol to each test tube. A 100 µl of each sample was separately injected on Gilson HPLC for analysis using Kromasil 100 C-18 column (10 µm, 25 x 0.46 mm). The absorbance was measured at 220 nm. A 1 % TFA solution was prepared in acetonitrile and mixed with water (70: 30 ratio) to prepare mobile phase with a flow rate of 1 ml/minute.

Statistical analysis: The experimental design was completely randomized. The data was analyzed using MSTAT and means were separated by LSD value at a 0.5% probability level.

Results and Discussion

Leaves of *Artemisia absinthium* contained 223 µg/g fresh weight of artemisinin while there was no detectable artemisinin present in the stem. Zhou & Xu (1989) reported that several US strains contain artemisinin with mean value ranging from 0.05% to 0.21% and several individual plants producing artemisinin up to 0.4% at the full flowering stage. A number of plant species from Northern Pakistan has been reported to contain artemisinin ranging from 0.337 to 0.435% (Mannan & Mirza, 2004).

The results given in Table 1 describe the effect of growth regulators on the production of artemisinin in the callus proliferated on liquid MS medium. Callus proliferated on MS medium without any growth regulator contained no artemisinin while callus proliferated on MS medium containing different concentrations of growth regulators showed different quantities of artemisinin. Cytokinins and auxins were found to be effective in increasing the artemisinin synthesis in callus culture. When 2.0mg/l BAP was added to the medium, 3.05 µg/g artemisinin was detected in the callus. However, when NAA and Kinetin were separately added to the medium at the same concentration, 1.95 µg/g and 1.7 µg/g artemisinin respectively, was produced. Whipky *et al*., (1992) reported that inclusion of BAP and Kinetin increased 3.6 and 2.6 times artemisinin concentration in callus culture respectively. These terpenoids are localized in the heart shaped glandular trichomes found in *Artemsia* species. These trichomes are formed after differentiation so there is possibility that trichomes are formed when callus was grown in contact form then in liquid medium on agitation.

Zhou & Xu (1989) reported that artemisinin increased upto 0.16% in shoot originated cultures maintained on MS medium supplemented with 0.2 mg/l GA_3 alongwith casein hydrolysate and nafitin, while other growth regulator had a negative effect when applied as elicitors and gene regulators. It is therefore suggested that increase or decrease in the artemisinin production is hormone specific and correlate with other factors like presence/absence of amino acids.

Table 1. Effect of different plant growth regulators on the quantity of artemisinin present in the callus cultured from leaf explant of *Artemisia absinthium*.

Plant growth regulator	Concentrations	Artemisinin (ug/g; FW)	Callus morphology
BA	1.0 mg/l	1.5^{BCD}	Lush green, hard, compact,
	2.0 mg/l	3.05^{A}	embryogenic
NAA	1.0 mg/l	1.05^{D}	Light green, compact,
	2.0 mg/l	1.95^{BC}	embryogenic, soft
IBA	1.0 mg/l	Undetected	Greenish brown, soft, friable
	2.0 mg/l	1.4^{CD}	
Kin	1.0 mg/l	0.85^{DE}	Dark green, compact,
	2.0 mg/l	1.7^{BC}	embryogenic

Mean separation by LSD α = 0.05, ^{abcd} Least significant difference value.

Table 2. Effect of different amino acids on the quantity of artemisinin present in the callus cultured from leaf explant of *Artemisia absinthium*.

Amino acids	Concentrations	Artemisinin (ug/g)	Callus morphology
Valine	2.5 mg/l	1.8^{BC}	Light green, hard, compact
	12.5 mg/l	3.1^{A}	
leucine	2.5 mg/l	0.22^{FG}	Greenish white, compact, nodular
	12.5 mg/l	0.58^{EF}	
Tyrosine	2.5 mg/l	Undetected	Yellowish green, friable, soft
	12.5 mg/l	0.9^{DE}	
Cystine	2.5 mg/l	0.85^{DE}	Green, compact, hard, embryogenic
	12.5 mg/l	2.8^{AB}	
Glycine	2.5 mg/l	0.35^{F}	Green with white margins, compact, well grown
	12.5 mg/l	0.9^{DE}	
Histidine	2.5 mg/l	Undetected	Yellowish, soft, watery, small increase in mass
	12.5 mg/l	0.045^{G}	
Glutamic acid	2.5 mg/l	Undetected	Yellowish in the centre while brown at margins,
	12.5 mg/l	0.3^{F}	soft
Lysine	2.5 mg/l	Undetected	Light green, soft, compact
	12.5 mg/l	1.6^{C}	
Threonine	2.5 mg/l	0.65^{E}	Light green, soft, compact, dark green at some where
	12.5 mg/l	2.3^{B}	
Phenylalanine	2.5 mg/l	Undetected	Whitish green, soft, compact
	12.5 mg/l	0.4^{EF}	
Serine	2.5 mg/l	0.45^{EF}	Light green, hard, margins lush green
	12.5 mg/l	1.85^{BC}	

Mean separation by LSD α = 0.05, ^{abcd} Least significant difference value.

Results pertaining to the effect of amino acid on the production of artemisinin in callus culture and morphological characteristics are given in Table 2. When 12.5 mg/l phenylalanine was added to the medium, callus showed the presence of 0.4 µg/g artemisinin. Addition of cystine and leucine 12.5 mg/l each, in the medium resulted in the production of 2.8 µg/g and 0.58 µg/g artemisinin respectively. Highest (3.1 µg/g) artemisinin was found in callus cultured on MS medium containing 12.5 mg/l of valine. Other amino acids like tyrosine, glutamic acid, histidine, proline, aspartic acid, etc. failed to stimulate the production of artemisinin in the callus. The results, therefore, suggest that

the production of artemisinin is amino acid specific. Although, there is no report available on the effect of amino acids on the synthesis of artemisinin in the callus culture of *Artemisia absinthium*, many authors described the biochemical basis of synthesis of other secondary metabolites in different plants. Boner (1972) suggested that some amino acids are incorporated into intermediate compounds of glycolysis and TCA cycle after deamination. Acetyl CoA is a precursor of secondary metabolites and is converted into isopentyl pyrophosphate which is precursor of terpenoids. Ibrahim (1987) found that the addition of phenylalanine into agar medium improved in rosmarinic acid yield in *Col. blumei* cell culture. Schripsema & Verpoorte (1997) reported that HMG CoA (3-hydroxy 3-methyl glutaryl coenzyme A) in plants is formed not only during the process of terpenoid biosynthesis but also as intermediate in leucine catabolism. Thus leucine serves as a direct progenitor of terpenoid. Since artemisinin is also a secondary metabolite it is plausible that amino acid stimulate its synthesis in callus culture. Amino acids also changed the morphological characteristics of callus. This may be due to production of some plant hormones under the influence of amino acids.

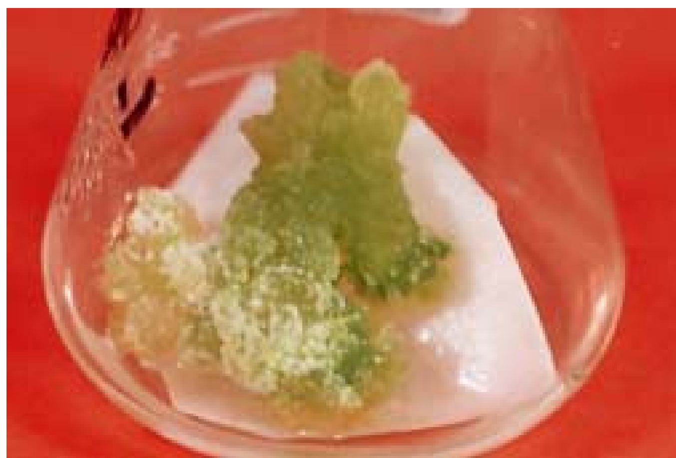

Fig. 1. Callus proliferation on Sterile-paper bridge on liquid MS medium supplemented with 12.5mg/l glycine.

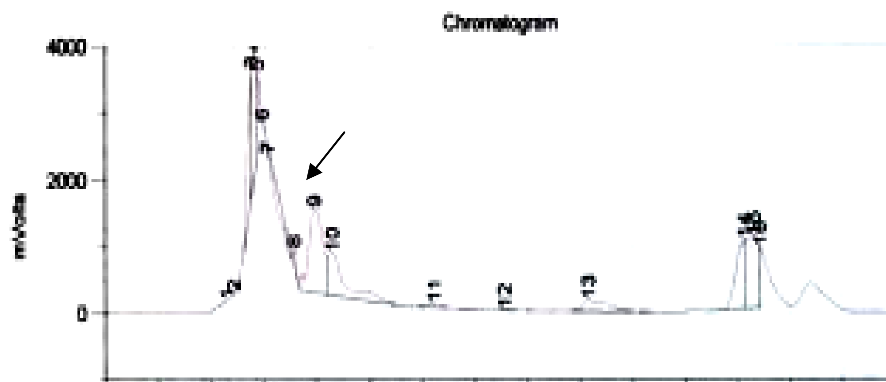

Fig. 2. Chromatogram for callus extract, grown on MS medium supplemented with 12.5mg/l valine, showing artemsinin peak (9).

The present study would suggest that manipulation of medium by adding different hormones and amino acids can enhance the production of artemisinin in the callus when cultured on sterile filter paper bridge. However on an average, the amount of artemisinin present in the leaves of plant is 40-50 times greater than in callus culture manipulated with growth hormones and amino acids.

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