Current Trends in Biotechnology and Pharmacy, Vol. 2 (2) 341-348 (2008)
ISSN 0973-8916

Enhanced artemisinin production by cell cultures of *Artemisia annua*

Ashish Baldi* [1] and V.K. Dixit [2]

[1] Department of Biochemical Engineering and Biotechnology,
Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
[2] Department of Pharmaceutical Sciences, Dr. H.S. Gour University, Sagar (M.P.) 470003, India

* For correspondence - baldiashish@gmail.com

Abstract

Artemisinin, an endoperoxide containing sesquiterpene lactone from *Artemisia annua*, has proven very effective in treating drug resistant cases of malaria and cancer. To counter the present low content in leaves and uneconomical chemical synthesis, alternate ways to produce artemisinin have been sought. Inspite of extensive work in this area, artemisinin remains elusive in dedifferentiated and differentiated cultures of *A. annua*. This work reports the first successful approach for production of artemisinin by cell cultures of *A. annua* cultures of Indian variety of *A. annua*. Various precursors of terpenoid biosynthesis by isoprenoid pathway were incorporated to study their influence on artemisinin biosynthesis. Artemisinin content was maximally increased by 2.0 times, in comparison to control, when mevalonic acid (50 mg/L) was added as precursor. Various biotic and abiotic elicitors were also tested at different concentration. A maximum increase of 3.47 times in artemisinin accumulation was attained when methyl jasmonate (5 mg/L) was added. Based on these results, an integrated bioprocess for productivity enhancement of artemisinin was developed. A maximum artemisinin accumulation of 96.8 mg/L artemisinin was produced on supplementation of mevalonic acid and methyl jasmonate as selected precursor and elicitor respectively, which was 4.79 times higher in productivity than control callus cultures (20.2 mg/L).

Keywords

Artemisinin, *Artemisia annua*, Hairy roots, *Agrobacterium rhizogenes*

Introduction

Malaria is a serious endemic disease in many parts of the world, affecting 5% of the World's population. About 40% of the world's population is at stake of malaria. In spite of availability of extensive chemotherapeutic armamentarium to combat this disease, widespread emergence of resistant strains of the parasite to drugs used is a global concern (1). A Chinese medicinal herb, *Artemisia annua* L., has evoked wide interest for its artemisinin (AN) content, a serquiterpene lactone, which is effective against both chloroquine-resistant and sensitive strains of *P. falciparum* as well as cerebral malaria with high safety profile. It is also reported as chemotherapeutic agent against various types of cancers such as leukemia and colon cancer etc. Significant biological activity and novel chemical structure have prompted efforts in developing a series of derivatives which are more potent than the parent compound, such as artesunate, artemether, arteether (2) and sodium salt of artelinic acid (3). Highest AN content has been reported in leaves (0.01-0.5%) in Chinese varieties of plant (4). It was later introduced in India, cultivated and naturalized in Kashmir and Lucknow (5) but showed low AN content. Uneconomical synthesis of AN (6)

and low yield from natural source (7) had triggered search for alternative means to produce AN in cell cultures. Results from experiments with undifferentiated and differentiated cultures of *A. annua* till now are rather disappointing with respect to the AN production, as only traces of this compound have been found (8-12) Till the date, highest AN content of 0.42% DW in hairy roots was reported by Weathers et al. (13). Few researchers reported a very low level (14,15) or absence of AN in hairy root cultures (16).

Feeding of precursors and elicitation has proved to be an effective way to enhance secondary metabolites in plant cell cultures. A number of precursors such as sodium acetate (17-19), mevalonic acid lactone, casein acid hydrolysate (20) and elicitors like jasmonates (21), salicylates (22), chitosan and gibberlic acid (20) have been investigated to enhance yield of plant based secondary metabolites. Effect of single precursor/elicitor on a particular cell line was investigated in most of these studies. But considering the complexicity of AN biosynthesis, it is reasonable to examine the combined effect of these yield enhancement strategies. For this reason, the present study was undertaken on cell suspension cultures of *A. annua*. The aim of this work was to develop a high yielding starter culture for AN production in cell cultures. This appears to be the very first successful study on production of AN in cell cultures developed from Indian variety of *A. annua*.

Material and Methods

Germination of seeds

Seeds of Indian variety of *Artemisia annua* were sterilized by treating first with 90% alcohol for 3 s, and followed by 0.1% (w/v) mercuric chloride treatment for 5 m. Seeds were then rinsed thoroughly 3 times in sterile distilled water and 10 seeds per Petri plate were allowed to germinate on padding of sterile filter paper and absorbent cotton, moistened with 15 ml of liquid Murashige and Skoog's (MS) medium (23) supplemented with 3% (w/v) sucrose. Seeds were then incubated at 25±2° under 2200 lux intensity of light and 16/8 h light/dark photoperiod in a growth chamber. Germination started within 2-3 d.

Callus cultures

High yielding callus culture of *A. annua* was developed from aseptically germinated seedlings and maintained on MS medium supplemented with NAA and Kn (0.5 mg/L of each) as growth regulators.

Establishment of growth and production kinetics

Growth and production kinetics of *A. annua* cell culture were established. Callus cultures were allowed to grow on MS media with 0.5 mg/L each of NAA and Kn, and solidified with 1% agar. Experiments were carried out in 250 mL Erlenemeyer flasks containing 50 mL of MS solid medium with 2.0 g/L inoculum on a dry weight basis. Cultures were incubated at 25±2°C under 16/8h light/dark cycle. All cultures were analyzed, in triplicate, for biomass and AN content at an interval of 5 d.

Enhancement of artemisinin production

Precursor addition

To improve AN production in callus cultures, some known precursors of AN biosynthesis [sodium acetate (SA), mevalonic acid (MA), casein acid hydrolysate (CAH)] and cholesterol (CH) were added. Stocks of sodium acetate and casein acid hydrolysate were prepared in sterile double distilled water and the pH was adjusted to 5.7. Cholesterol and mevalonic acid were dissolved in acetone and 95% ethanol respectively. Filter sterilized precursors were added to the culture medium on 10^{th} d at following concentrations: sodium acetate, 20, 50 and 100 mg/L; mevalonic acid,

20, 50 and 100 mg/L; cholesterol, 20, 50 and 100 mg/L; casein acid hydrolysate, 0.1, 0.5 and 1.0 g/L. Cultures were harvested on 25th d, in duplicate, and analyzed for biomass and AN content. In control cultures, equal volume of solvent for precursor was added aseptically to culture medium.

Elicitor treatment

Some known abiotic elicitors for plant based secondary metabolites such as signaling molecules [(methyl jasmonate (MJ), acetyl salicylic acid (ASA)], metal ion [calcium as calcium chloride (CC)] and blooming agent [gibberlic acid (GA)] were also tested for their effect on AN content. Stock solutions of acetyl salicylic acid, and calcium chloride were prepared by dissolving them in sterile double distilled water and adjusting the pH to 5.7. Methyl jasmonate was dissolved in 95% ethanol. Abiotic elicitors were aseptically added to cultivation medium at the following concentrations: methyl jasmonate: 1,2,5 and 10 mg/L; acetyl salicylic acid: 10, 20, 30, and 50 mg/L; calcium chloride: 50,100 and 150 mg/L; and gibberlic acid: 5, 10 and 20 mg/L.

Biotic elicitors [yeast extract (YE), chitosan (Ch)] were tested at 10, 20, and 50 mg/L concentrations by addition of 50 µL, 100 µL and 250 µL of respective stock solutions of yeast extract and chitosan individually.. Yeast extract was added as aqueous stock (10 mg/ml) having pH 5.7. For preparation of chitosan, 1 g of crab shell chitosan was dissolved in 2 ml of glacial acetic acid (by dropwise addition) at 60°C for a period of 15 minutes.; the final volume was made up to 100 ml with water and pH of the solution was adjusted to 5.7 with 1 M NaOH to get a stock solution of 10 mg/ml.

Following the elicitor treatment on 20th day, cultures were harvested on 25th day, in triplicate, and analyzed for biomass and AN content.

Integrated bioprocess for productivity enhancement

Results from both yield enhancement strategies *viz.* addition of precursor and elicitation were integrated in order to develop a bioprocess for synergistic enhancement of artemisinin productivity in callus cultures of *A. annua*. Cultures were cultivated on solid MS medium with NAA and Kn (0.5 mg/L of each) at 27±2°C with a photoperiod of 16/8h light/dark cycle). Selected precursor (mevalonic acid @ 50 mg/L) and elicitor (methyl jasmonate @ 5 mg/L) were added on 10th and 20th day of cultivation respectively. Cultures were harvested in triplicate on 25th day and analyzed for biomass and AN production.

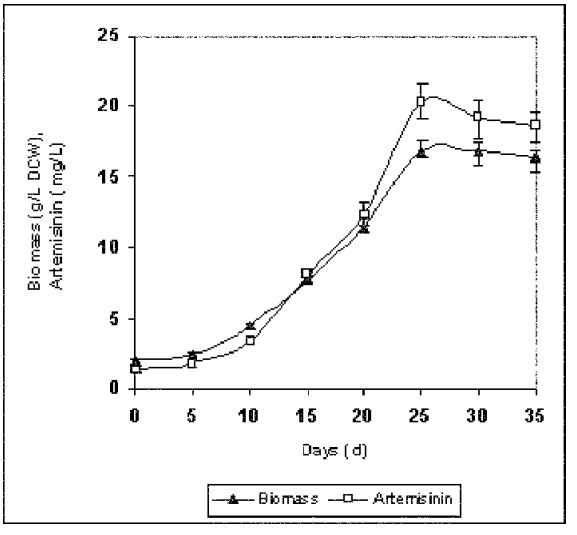
Analysis

For dry cell weight estimation, cells were harvested and allowed to dry at 60±2°C until constant weight was achieved. Sodium derivative of AN was analyzed by HPLC with some modification in the method of Zhao and Zheng (24). For analysis of AN, 100 mg of dried and powdered cells were allowed to macerate with 2.0 mL diethyl ether for 48 h. The filtrate was then dried under vacuum and the residue was dissolved in 1.0 mL of methanol. This was then treated with 4.0 ml NaOH (0.2% w/v) and incubated at 50±2°C for one h with occasional shaking to get a sodium derivative of AN.

Results and Discussion

Cell cultures of *A. annua* of Indian variety with significant AN accumulation were established for very first time. Complete growth and production profiles of *A. annua* cells were established and given as Fig. 1. Relatively longer log phase of 10 day was observed in batch kinetics. Maximum biomass (16.8 g/L) and artemisinin (20.2 mg/L) were obtained on 25th day of cultivation. Artemisinin was produced in callus cultures.

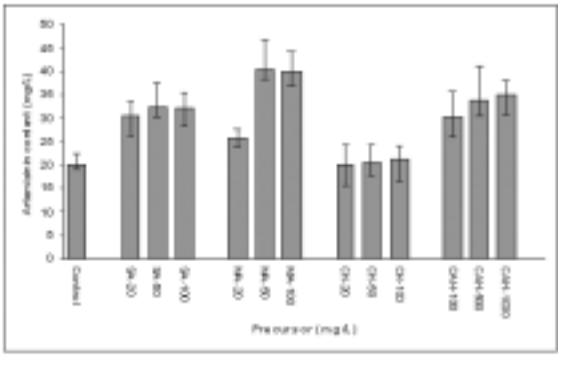
Figure 1 Growth and artemisinin production profile of *A. annua* cells (Average values are given, error bars are represented as vertical lines**)**



intracellularly and most production occurred during the active growth phase of *A. annua* cells.

Few known precursors for terpenoid biosynthesis were added to the media on 10th d of cultivation. Their effect on AN production is given in Fig. 2. Sodium acetate, mevalonic acid and higher concentration of casein acid hydrolysate had positive effect on AN production

Figure 2 Effect of precursor addition on artemisinin production by *A. annua* callus cultures (Average values are given, error bars are represented as vertical lines)



A maximum of 32.2 mg/L artemisinin was produced on addition of 50 mg/L sodium acetate. It is known to be a potential precursor for various secondary metabolites. It has been found to increase secondary metabolites like azadirachtin (25); astaxanthin (26); taxol (17,18,27); crocin (19) and phenylethnoid glycosides (28). The increased production of AN is assumed to be attributed to the increased Acetyl-Co A level as a result of sodium acetate feeding, which may lead to the improved secondary metabolite production by enhanced life activities (29).

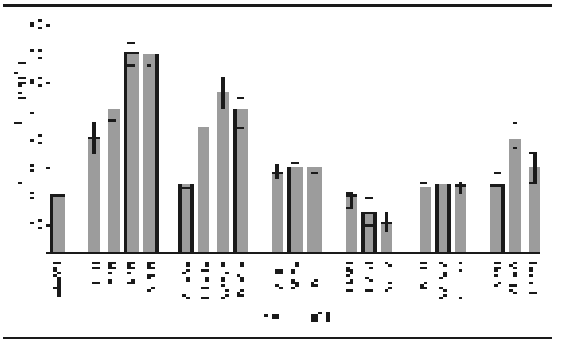
Mevalonic acid is an intermediate in the biosynthesis of isoprene units. AN content was increased maximally by 2.0 times (40.4 mg/L), in comparison to control, when mevalonic acid (50 mg/L) was added. However, this is not in agreement with earlier findings (8). This enhancement might be due to direct supply of mevalonic acid to synthesize AN at enhanced level.

Casein acid hydrolysate serves as an additional source of amino acids and oligopeptides. With 0.5 g/L, a slight enhancement of the AN content was found, but it remains almost constant after this. Indirect precursor, cholesterol, had a negative effect on biomass as well as AN production. This might be due to diversion of metabolic pathway to other compounds.

Various biotic (chitosan and yeast extract) and abiotic elicitors (gibberlic acid, calcium chloride, acetyl salicylic acid and methyl jasmonate) were also tested at different concentration to enhance AN yield. Effect of these elicitors on AN accumulation is given in Fig. 3. Acetyl salicylic acid, methyl jasmonate, and gibberlic acid had resulted in significant improvement of AN production.

Gibberlic acid is a plant growth regulator, capable of inducing blooming (30). AN content

Figure 3 Effect of elicitor addition on artemisinin production by *A. annua* callus cultures (Average values are given, error bars are represented as vertical lines)



in intact plant are maximum just before or at the time of flowering (31), so this compound was added to callus cultures. Earlier, addition of gibberlic acid has been reported not to affect the AN content of *A. annua* shoot cultures (8) but a positive effect was also observed (20).

Acetyl salicylic acid also significantly improved AN production as a maximum increase of 2.81 times (56.8 mg/L) was attained when acetyl salicylic acid (30 mg/L) was added. This could be attributed to the fact that salicylic acid and related compounds act as signaling molecules and play essential role in many plant defence reactions (32). It is known to regulate the expression of various defense related genes (33,34) and has an important role in System Acquired Resistance (SAR) and inhibitor of ethylene biosynthesis (35). AN being the defense chemical of plants could be regulated with the presence of these signal molecules.

Addition of methyl jasmonate resulted in an increased AN production of 70.2 mg/L at 5.0 mg/L concentration. Exogenous application of JA signaling compounds, including jasmonic acid, methyl jasmonate, as well as their conjugated compounds to the plant cell culture or intact plant stimulates biosynthesis of secondary metabolites (36-38). Elicitor-induced indole alkaloids in *C. roseus* (39), phytoalexin biosynthesis in rice (40), indole glucosinolates biosynthesis in Arabidopsis (41), and h-thujaplicin in Mexican cypress cell culture (42) support the idea that JA signaling is a mediator of secondary product accumulation. Chemical structure of jasmonic acid related molecule also known to play an important role in elicitation and is very specific in nature (43).

Addition of yeast extract also slightly enhanced AN levels. The possible reason could be attributed to presence of some cations like ZN and Co (44), which could act as abiotic elicitors. Although addition of calcium ions did not improve AN content in callus cultures. A reduction in the AN content was observed by addition of chitosan. This might be due to change in cell membrane permeability as evident from browning of cells.

Based on these results, an integrated bioprocess for synergistic enhancement of artemisinin was developed and experimentally implemented. A maximum of 96.8 mg/L artemisinin was produced on supplementation of mevalonic acid lactone (50 mg/L on 10^{th} day) and methyl jasmonate (5 mg/L on 20^{th} day) as selected precursor and elicitor respectively, which was 4.79 times higher in productivity than control cultures. A comparative effect of yield enhancement strategies tried out in present study is given in Table-1**.**

Table 1: Comparison of yield enhancement strategies

Yield enhancement strategy	**Artemisinin production (mg/L)**
High yielding cell line selection	20.2
Precursor addition	40.4
Elicitation	70.2
Integrated bioprocess	96.8

Conclusion

This study represents the first successful cell culture based approach for the production of artemisinin from Indian variety of seeds of *A. annua*. Artemisinin content was found to be superior to that reported earlier (11,15). The problem of low content in plant cell cultures was addressed through addition of precursor and elicitation, which resulted in significant improvement in artemisinin production. Integrated yield enhancement strategy comprising of addition of mevalonic acid as precursor and methyl jasmonate as elicitor at optimum concentrations resulted in synergistic enhancement of artemisinin accumulation. The present study indicates the potential of these biotechnology-based methodologies for mass production of artemisinin. Further work related to large scale production of artemisinin at bioreactor level under different configurations and various modes of cultivation are under progress.

Acknowledgements

The authors are thankful to Prof. Pamela Weathers, Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, USA for her valuable guidance and generous gift of standard artemisinin.

References

1. White, N. and Pukrittayakamee, J. (1993) Clinical malaria in tropics, S. Med. J. Aust., 159: 197-203.
2. Titulaer, H.A.C., Zuidema, J. and Lugt , C.B. (1991) Formulation and pharmacokinetics of artemisinin and its derivatives, Inter. J. Pharm., 69, 83–92.
3. Lin, A., Klayman, D.L. and Milhous, W.K. (1987). Antimalarial activity of new water soluble dihydroartemisinine derivatives. J. Med. Chin., 30: 2147–2150.
4. Nair, M.S.R., Acton, N., Klayman, D.L., Kendrick, K., Basile, D.V. and Mante, S. (1986). Production of artemisinin in tissue culture of *Artemisia annua*. J. Nat. Prod., 49: 504-507.
5. Singh, A., Vishwakarma, R.A. and Husain, A. (1988). Evaluation of *Artemisia annua* strains for higher artemisinin production. Planta Med., 54: 475-476.
6. Avery, M.A., Chong, W.K.M. and Jennings-White, C. (1992). Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. J. Am. Chem. Soc., 114: 974-979.
7. E1Sohly, H.N., Croom Jr, E.M., El-Feraly, F.S. and El-Sherei, M.M. (1990). A large-scale extraction technique of artemisinin from *Artemisia annua*, J. Nat. Prod., 53: 1560-1564.
8. Martinez, B.C. and Staba, E.J. (1988). The production of artemisinin in *Artemisia annua* L. tissue cultures. Adv. Cell Cult., 6, 69-87.
9. He, X.C., Zeng, M.Y., Li, G.F. and Liang, Z. (1983). Callus induction and regeneration of plantlets from *Artemisia annua* and changes in qinhaosu contents. Acta Bot. Sin., 25: 87-90.
10. Kudakasseril, G.J., Lam, L. and Staba, E.J. (1987). Effect of sterol inhibitors on the incorporation of ~4C-isopentenyl pyrophosphate into artemisinin by a cell-free system from *Artemisia annua* tissue cultures and plants. Planta Med., 53: 501-502.
11. Jha, J., Jha, T.B. and Mahato, S.B. (1988). Tissue culture of *Artemisia annua* L.: A potential source of an antimalarial drug. Curr. Sci., 57: 344-346.
12. Tawfiq, N.K., Anderson, L.A., Roberts, M.F., Phillipson, J.D., Bray, D.H. and Warhurst, D.C. (1989). Antiplasmodial activity of *Artemisia annua* plant cell cultures. Plant Cell Rep., 8: 425-428.
13. Weathers, P., Cheetham, R.D., Follansbee, E. and Tesh, K (1994). Artemisinin production by transformed roots of *Artemisia annua*. Biotech. Lett., 16: 1281-1286.
14. Jaziri, M., Shimomur, A.K. and Yoshimatsu, K. (1995). Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. J Plant Physiol., 145: 175-177.

15. Paniego, N.B. and Giulietti, A.M. (1996). Artemisinin production by *Artemisia annua* L. transformed organ cultures. Enz. Microb. Tech., 18: 526-530.

16. Kim, N.C., Kim, J.G., Lim, H.J., Hahn, T.R. and Kim, S.U. (1992). Production of secondary metabolites by tissue culture of *Artemisia annua* L.J. Korean Agri Chem Soc., 35, 99-105.

17. Wu, Z.L., Yuan, Y.J., Liu, J.X., Xuan, H.Y., Hu, Z.D., Sun, A.C. and Hu, C.X. (1999). Study on enhanced production of taxol from *Taxus chinensis* var. *mairei* in biphasic-liquid culture. Acta Bot. Sin., 41: 1108-1113.

18. Yuan, Y.J., Wei, Z.J., Wu, Z.L. and Wu, J.C. (2001). Improved taxol production in suspension cultures of *Taxus chinensis* var. *mairei* by *in situ* extraction combined with precursor feeding and additional carbon source introduction in an airlift loop reactor. Biotechnol. Lett., 23: 1659-1662.

19. Zeng, Y., Yan, F., Tang, L. and Chen, F. (2003). Increased crocin production and induction frequency of stigma-like structure from floral organs of *Crocus sativus* by precursor feeding. Plant Cell Tiss. Org. Cult., 72: 185-191.

20. Woerdenbag, H.J., Lfiers, Jos F.J., van Uden, W., Pras, N., Malingr, Th..M. and Alfermann, A.W. (1993). Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua L.* Plant Cell Tiss. Org. Cult., 32: 247-257.

21. Walker, T.S., Bais, H.P. and Vivanco, J.M. (2002). Jasmonic Acid induced hypercin production in *Hypericum perforatum* L. (St. John wort). Phytochem., 60: 289-293.

22. O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, DJ. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science, 274: 1914-1917.

23. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant., 51: 473-497.

24. Zhao, S.S. and Zeng, M.Y. (1985). Studies on the analysis of qinghaosu by high-pressure liquid chromatograph and spectrometry (HPLC). Planta Med., 51: 233-237.

25. Balaji, K., Veeresham, C., Srisilam, K. and Kokate, C. (2003). Azadirachtin, a Novel biopesticide from cell cultures of *Azadirachta indica*. J Plant Biotechnol., 5: 121-129.

26. Orosa, M., Franqueira, D., Cid, A. and Abalde, J. (2001). Carotenoid accumulation in *Haematococcus pluvialis* in mixotrophic growth, Biotechnol. Lett., 23: 373-378.

27. Fett-Neto, A.G., Zhang, W.Y. and Dicosmo, F. (1994). Kinetics of taxol production, growth and nutrient uptake in cell suspensions of *Taxus cuspidata.* Biotechnol. Bioeng., 44: 205-210.

28. Ouyang, J., Wang, X.D., Zhao, B. and Wang, Y.C. (2005). Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of *Cistanche deserticola*. Proc. Biochem., 40: 3480-3484.

29. Lu, D.P., Zhao, D.X., Huang, Y. and Zhao, Q. (2001). The effect of precursor feeding on flavonoids biosynthesis in cell suspension cultures of *Saussurea medusa*. Acta Bot Yunnanica., 23: 497-503.

30. Evans, F.J. (1989). Trease and Evans' Pharmacognosy, Balliire Tindall, London.

31. Woerdenbag, H. J., Pras, N., Bos, R., Visser, J.F., Hendriks, H. and Malingr, Th.M. (1991). Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua L.* by combined gas chromatography/mass spectrometry. Phytochem. Anal., 2: 215-219.

32. Durner, J., Shah, J. and Kleesing, G.F. (1997). Salicylic acid and disease resistance in plants, Trends Plant. Sci., 6: 266-274.

33. Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science. 250: 1001-1004.

34. Shualev, V., Leon, J. and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco?, Plant Cell, 7: 1691-1701.

35. Dong, X. (1998). SA, JA, ethylene and disease resistance in plants. Curr. Opin. Plant Biol., 1: 316-323.

36. Gundlach, H., Muller, M.J., Kutchan, T.M. and Zenk, M.H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. U. S. A. 89: 2389– 2393.

37. Mueller, M.J., Brodschelm, W., Spannagl, E. and Zenk, M.H. (1993). Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc. Natl. Acad. Sci. U.S.A. 90: 7490–7494.

38. Tamogami, S., Rakwal, R. and Kodama, O. (1997). Phytoalexin production elicited by exogenously applied jasmonic acid in rice leaves (*Oryza sativa* L.) is under the control of cytokinins and ascorbic acid. FEBS Lett., 412: 61–64.

39. Menke, F.L.H., Parchmann, S., Mueller, M.J., Kijne, J.W. and Memelink, J. (1999). Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol., 119: 1289–1296.

40. Nojiri, H., Sugimori, M., Yamane, H., Nishimura, Y., Yamada, A. and Shibuya, N. (1996). Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. Plant Physiol., 110: 387–392.

41. Brader, G., Tas, E. and Palva, E.T. (2001). Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen *Erwinia carotovora.* Plant Physiol., 126: 849–860.

42. Zhao, J. and Sakai, K. (2001). Multiple signaling pathways mediate fungal elicitor induced h-thujaplicin accumulation in *Cupressus lusitanica* cell cultures. J. Exp. Bot., 54: 647–656.

43. Wang, W. and Zhong, J.J. (2002). Manipulation of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by addition of jasmonates. J. Biosci. Bioeng., 93: 48-53.

44. Suzuki, T., Mori, H., Yamame, T. and Shimuzu, S. (1985). Automatic supplementation of minerals in fed-batch culture to high cell mass concentration. Biotech. Bioeng., 27: 192-201.