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RESEARCH ARTICLE

Invitro Production of Secondary Metabolites from *Artemisia vulgaris*

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Abstract

Herbal medicine has been the basis of treatment for various diseases in traditional systems like Ayurveda. An increase in demand of ayurveda leads to overexploitation of medicinally imporant plant species. *Artemesia vulgaris* is an herb with high medicinal value and contains compounds of commercial importance. With an increase in use, plants need to be produced on a large scale. Tissue culture serves as the best method for mass production of the plant *via* callus culture or production of secondary metabolites through development of suspension culture. *A.vulgaris* leaf explants were cultured *in vitro* in MS medium supplemented with 2, 4-D 3mg/l to produce 90% callus in 15 days. Suspension culture was initiated from well grown callus in MS media. Supplementation of the media with 15 mg/l methyl jasmonate and 15mg/l acetyl salicylic acid produced artemisinin in the culture filtrate as confirmed by LC-MS. The present study indicates the feasibility of production of secondary metabolites of commercial value from *A. vulgaris* through suspension culture.

Keywords: *Artemesia vulgaris, Suspension culture, Methyl jasmonate, Artemisinin*

Introduction

Plants produce a plethora of secondary metabolites that play a significant role in the defense mechanism of the plant. Many of these secondary metabolites possess significant bioactivities. Medicinal plants are a treasure trove of bioactive principles with therapeutic properties. There is a constant search for newer molecules of therapeutic value from the plant kingdom. India is blessed with rich biodiversity and abounds in medicinal plants with diverse properties.The genus Artemisia is the largest and the most widely distributed member of the family Asteraceae. Members of *Artemisia* are found growing wild and abundantly throughout the temperate and cold-temperate zones of the world. *Artemisia vulgaris,* commonly called mugwort or common wormwood, is one of the several species in the genus. *Artemisia vulgaris* is also known as riverside worm wood, felon herb, chrysanthemum weed etc. Mugwort has been used medicinally and as culinary herbs in Ayurveda. Aerial parts of *A. vulgaris* are used in traditional herbal medicine. Antihelminitic activity, (Meschler and Howlett 1999), anti-malarial activity (Tu 2011), antibacterial activity (Chen *et al.,* 1989), hepatoprotective activity (Gilani, 2005) of *A.vulgaris* has been well demonstrated. Recently Tu reported avermectin and artemisinin from *Artemesia annua* that has revolutionized therapy for patients suffering from malaria for which she was awarded Nobel prize in Physiology and Medicine during 2015. Anti-inflammatory and anti oxidant activity of *A. vulgaris* leaf extracts has been studied in our lab (unpublished).

Secondary metabolites are the organiccompounds that are not involved in growth, development or reproduction of an organism. Secondary metabolites play a major role in the plant defense mechanism. The identified metabolites of *A. vulgaris* include flavonoids, coumarins, sesquiterpene lactones, volatile oils, inulin, and traces of alkaloids. The major compound present in *A. vulgaris* is artemisinin, asesquiterpene lactone having endoperoxide group (Krungkrai 2016) which plays a major role in the anti-malarial activity (White 2008). Dihydroartemisin is the active metabolite of all the artemisinin compounds (artemisinin, artesunate, artemether etc.). The plant contains many active compounds including the monoterpenes, eucalyptol, camphor, linalool, thujone, 4-terpineol, borneol and 21 other compounds. Monoterpenes are present in the essential oil that make up 0.03%-0.3% of the plant dry weight. The plant also contains sesquiterpenes and sesquiterpene lactones such as eudesmanevulgarin, psilostachyin.

At present artemisinin is available in large enough quantities only in China, where this plant grows

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abundantly, for extensive clinical trials and treatment. Compounds received from plants or *in vitro* cell cultures may be directly used as drugs, without any changes or such compounds may undergo further semi synthetic modifications (Bourgaud *et al.,* 2001). Seasonal and regional differences influence artemisinin content and availability. To ensure year round availability and predictable content of artemisinin, we have attempted callus induction and *in vitro* production of secondary metabolite artemisinin from suspension cultures of *A. vulgaris* L.

Material and Methods

Collection of plant sample

Artemisia vulgaris L. plants were collected from areas in Coimbatore. The plant was validated by Botanical Survey of India (BSI), Coimbatore (No. BSI/SRC/5/23/2016/ Tech. /277) and a voucher specimen is maintained in the Department of Biochemistry. Plants were maintained in the green house of Dept. of Biotechnology, CPMB&B, Tamil Nadu Agricultural University (TNAU), Coimbatore.

Preparation of explants

Leaves of *A.vulgaris* L. were collected from plants maintained in the green house and used as explant for callus induction and maintenance of suspension culture.Young healthy leaves of *A.vulgaris* L. were washed under running water for 3-5 times to remove the dirt and cut in to medium sized pieces. Cut bits of the leaf were washed with tween 20 (2-3 drops) and cleaned in running tap water, followed by transfer to laminar air flow cabinet and washing with distilled water (2-3 times). After this, the leaf bits were treated with 70% ethanol for less than 60 seconds, immediately followed by 1% $HgCl_2$ for 5 minutes. After 10 minutes,the explants were thoroughly rinsed in sterile water for 4 times. Leaf bits were blot dried in sterile tissue paper inside the laminar air flow chamber. These leaf bits were placed on the Murashige and Skoog media containing different concentrations of the hormones for the callus initiation.

Effect of plant growth regulators on callus induction

MS medium supplemented with growth regulators BAP, NAA and 2,4-D at varied concentration levels were studied for callus induction. 2, 4-Dicholro phenoxy acetic acid at 1.0, 2.0 and 3.0 mg/L, NAA at 1.0, 2.0 and 3.0 mg/L and combinations of BAP and NAA (0.75 mg/L and 0.5 mg/L), (1.5 mg/L and 1.0 mg/L), (2.0 mg/L and 1.5 mg/L) and (2.5 mg/L and 1.0 mg/L) (Ganesan *et al.,* 2011) were used to attempt callus induction from leaf explants. All cultures were maintained at 25±2°C at relative humidity of 60-70% under dark condition for callus induction from leaf explants. Sub culturing of calli was carried out at every three weeks interval on the same medium.

Callus induction frequency

The best treatment was decided based on the callus induction frequency (expressed in percentage) as recorded after 28 days of inoculation. Callus induction frequency was calculated using the formula given below.

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of explants showing initiation}}{\text{Total no. of explants inoculated}} \times 100$$

Suspension culture

Cell suspensions were initiated with soft friable mass of calli. Callus of 1g fresh weight was aseptically transferred in to 100 mL conical flasks containing 25mL of Murashige and Skoog medium supplemented with 3 mg L^{-1} of 2,4-D, 3% sucrose for proliferation. Suspensions were established by shaking the cultures in a rotary shaker at 100 rpm at 24°C under 16 h photoperiod provided by cool white fluorescent lamps. Three passages were done at weekly intervals. The elicitors methyl jasmonate and acetyl salicylic acid were added 15 days after initiation of suspension culture. Methyl jasmonate and acetyl salicylic acid were added in combinations of (5.0mg $L^{-1}$and 5.0mg L^{-1}), (10.0mg $L^{-1}$and 5.0mg L^{-1}), (5.0mg L^{-1} and 10.0mg L^{-1})and (15.0mg L^{-1} and 15mg L^{-1}). (Baldi*etal.,* 2008) Seven days after the addition of elicitors, the culture filtrate was tested for the presence of secondary metabolites by TLC. This was followed by analysis in Shimadzu LC-MS-MS 8040 in a mass range of 2 to 2000m/z using methanol: acetonitrile: water in the ratio 20:50:30 as the solvent system.

Results and Discussion

Callus induction

To assess the effect of different plant nutrient media and growth regulators on callus induction, leaf explants of *A. vulgaris* L. were cultured on full MS medium supplemented with growth hormones BAP, NAA and 2,4-D (Table 1).

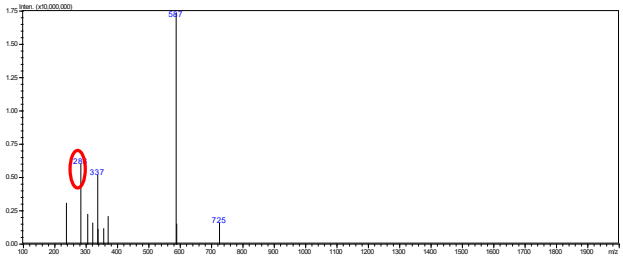
Table 1. Callus induction in A. vulgaris L. leaf explants

Growth regulator (mg/l)			Days required for callus formation	Callus Induction (%)	Colour of callus
BAP	NAA	2,4-D			
-	-	1.0	15	60	Green
-	-	2.0	15	80	Green
-	-	3.0	15	90	Green
-	0.5	-	20	70	Green
-	1.0	-	20	60	Green
-	1.5	-	20	50	Green
0.75	0.5	-	25	50	Dark green
1.5	1.0	-	25	50	Dark green
2.0	1.5	-	25	60	Dark green
2.5	1.0	-	20	70	Dark green

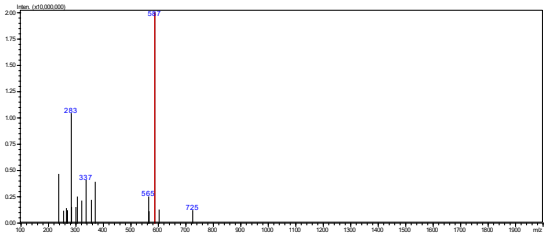
In media supplemented with 2,4-D, callus induction was observed in 15 days, producing green coloured callus. 2,4- D at 3.0 mg/L was the best combination resulting in 90% callus formation. MS medium supplemented with 0.5, 1.0 and 1.5 mg/L of NAA responded in 20 days producing green colour callus. Among these

Artemisia vulgaris callus culture suspension culture

A. Mass spectrum of sample



B. Mass spectrum of standard artemisinin



LC-MS- MS chromatograph of suspension culture filtrate

A) Positive ionization showing peak at 283 in sample

B) Positive ionization showing peak at 283 in standard

combinations, NAA at 0.5 mg L^{-1} reported highest callus induction of 70%. Supplementation of media with different combinations of BAP and NAA as detailed in Table 1 resulted in production of dark green callus in 25 days.The combination of BAP at 2.5 mg L^{-1} and NAA at 1.0mg L^{-1}resulted in 70% callus induction. Dry weight

% of callus was recorded at 7 days intervals as 10.0± 0.13% at 14 days, 16.0± 0.24 % at 21 days and 25.0± 0.76% at 28 days.Callus initiated on media supplemented with 2, 4-D at 3 mg/L recorded dry weight of 16.0±0.24 % at 21 days after subculture. Several works on callus initiation in different *Artemesia* species have been reported (Kumar, 2010;Mohiuddin and Nathar, 2018). they have attempted callus induction from *A. vulgaris* leaves in MS, ½ MS and B5 medium and concluded MS medium supplemented with 0.5 ppm 2, 4-D to be the best, recording 81.4% callus induction. High callogenic frequency of 85% has been observed in shoot tip explants of *A. vulgaris* in MS media supplemented with 0.75mg/L kinetin (Nathar *et al.* 2014).

Suspension culture

In vitro culturing of *Artemesia* species including *A. vulgaris* have been attempted by many workers for the production of biologically active secondary metabolites (Borzabad *et al*., 2010). Attempts have been made to increase the production of artemisinin using elicitors. Most workers attempted yield enhancement of artemisinin through exogenous application of methyl jasmonate as elicitor (Wang *et al.,* 2010; Xiang *et al*., 2015). Cell suspension culture was established from healthy soft, friable mass of callus maintained in MS medium with 3 mg/L of 2,4-D. Suspension was maintained in basal MS medium with growth regulator (3mg/L of 2,4-D). Effect of growth regulator on % of packed cell volume (PCV) and cell growth over three passages were determined (Table 2). In suspension culture, minute cells were separated uniformly. Packed cell volume (PCV) and dry weight percentage of suspension was calculated at 7th day, 14th day and 21st day of subculture. PCV values of basal medium were found to be increasing from 3.42% to 5.26% in II passage and to 7.46% in III passage. Elicitors,methyl jasmonate and acetyl salicylic acid were added after the second passage *i.e*.15 days after initiation of suspension culture. The presence of artemisinin in the culture filtrate was checked after 10 days by TLC.

Detection of secondary metabolites in suspension

The combination of 15.0 mg/L of methyl jasmonate and 15.0 mg/L of acetyl salicylic acid resulted in artemisinin production as detected by TLC. Suspension culture was further analyzed by LC-MS-MS for detection of secondary metabolites (Fig.1). The results gave a chromatograph showing the presence of compound in suspension culture filtrate. The ionization peak at 283 indicates artemisinin monomer. The peak at 567 could be the dimer of artemisinin. The other peaks observed may be due to the growth regulators and media components in the suspension filtrate. The LC-MS-MS report shows significant production of artemisinin in the suspension culture. Wang *et al.* (2010) reported 50% increase in artemisinin derivative and 65% increase in secondary metabolites on application of methyl jasmonate in *A. annua*. Guo *et al*., (2010) have attributed the enhancement of artemesinin production elicited by salicylic acid and methyl jasmonate to the invocation of burst of endogenous singlet oxygen that facilitated enhancement in artemisinin production from suspension cultures.The present study has indicated the possibility of enhancing production of artemisinin in suspension cultures of *A. vulgaris* L.

Conclusion

The present study has proven the feasibility of production of artemisinin in suspension cultures of *A. vulgaris* L. Improvement in artemisinin content, minimizing other components in suspension culture and scaling up of the system for large scale production of artemisinin in reactors could make *in vitro* production of artemisinin commercially viable.

References

Baldi, A. and V.K Dixit.2008."Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*". *Bioresource technology*, **99(11)**: 4609-4614.

Borzabad, R. K.,M. Sudarshana and M.H. Niranjan. 2010. "In vitro plant regeneration from leaf explants of *Artemisia vulgaris* L.–a medicinal herb". *Modern Applied Science,* **4(9)**:130.

Bourgaud, F., A. Gravot, S.Milesi and E.Gontier. 2001. "Production of plant secondary metabolites: a historical perspective". *Plant science,* **161(5)**: 839-851.

Chen, C. P.,C. C. Lin and N.Tsuneo. 1989."Screening of Taiwanese crude drugs for antibacterial activity against *Streptococcus* mutants". *Journal of ethnopharmacology*, **27(3):** 285-295.

Ganesan, C., and Paulsamy, S. (2011). Standardized protocol for the in vitro culture of Artemisia annua L.A medicinal plant at high altitudes of Nilgiris, the Western Ghats. *Journal of research in Biology,* **3**, 173-178.

Gilani, A. H., S. Yaeesh, Q. Jamal and M. N. Ghayur. 2005."Hepatoprotective activity of aqueous-methanol extract of *Artemisia vulgaris*". *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, **19(2)**: 170-172.

Guo, X.X., X.Q. Yang, R. Y. Yang and Q.P. Zeng. 2010. "Salicylic acid and methyl jasmonate but not Rose Bengal enhanceartemisinin production through invoking burst of endogenous singlet oxygen". Plant Science, 178(4):

390-397.

Krungkrai, J. and S.R.Krungkrai. 2016. "Antimalarial qinghaosu/artemisinin: the therapy worthy of a Nobel Prize". *Asian Pacific Journal of Tropical Biomedicine*, **6(5)**: 371-375.

Kumar, S. P. and B.R. Kumari. 2010."Effect of amino acids and growth regulators on indirect organogenesis in Artemisia vulgaris L". *Asian Journal of Biotechnology*, **2(1)**: 37-45.

Meschler, J.P. and A.C. Howlett. 1999."Thujone exhibits low affinity for cannabinoid receptors but fails to evoke cannabimimetic responses". *Pharmacology Biochemistry and Behavior*, **62(3)**: 473-480.

Mohiuddin, Y.G. and N.V. Nitin.2016."*In-vitro* studies of a valuable medicinal plant-*Artemisia vulgaris* L.(mugwort)". *World Journal of Pharmacy and Pharmaceutical Sciences*, 5(4): 1203-1210.

Mohiuddin, Y. G., V. N. Nathar, W. N. Aziz and N. B. Gaikwad. 2018. "Induction of callus and preliminary phytochemical profiling from callus of *Artemisia absinthium* L. and *Artemisia pallens* Wall". *International Journal of Current Trends in Science and Technology*, **8(2)**: 20236-20241.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, **15(3)**, 473-497

Nathar, V. N., and Yatoo, G. Mohiuddin (2014).Micropropagation of an antidiabetic medicinal plant, *Artemisia pallens. Turkish Journal of Botany,* **38(3)**, 491-498.

Tu, Y. 2011. "The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine".*Nature medicine*, **17(10)** :1217.

Wang, J., L.Huang, J.Li, Q.Fan, Y. Long, Y. Li and B. Zhou. 2010."Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation".*Plos one*, **5(3)**: 9582.

White, N.J. 2008. "The role of anti-malarial drugs in eliminating malaria". *Malaria journal,* **7(1)**: S8.

Xiang, L., Zhu, S., Zhao, T., Zhang, M., Liu, W., Chen, M. and Liao, Z. (2015). Enhancement ofartemisinin content and relative expression of genes of artemisinin biosynthesis in *Artemisia* annua by exogenous MeJA treatment. Plant growth regulation,**75(2)**, 435-441.

Yuan, Y., W.Liu, Q.Zhang, L.Xiang, X. Liu, M. ChenandZ and Liao. 2015."Overexpression of artemisinic aldehyde 11 (13) reductase gene-enhancedartemisinin and its relative metabolite biosynthesis in transgenic *Artemisia annua* L".*Biotechnology and applied biochemistry*, **62(1):**17-23.