Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):303-310

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization of *in vitro* regeneration of *Artemisia absinthium* L. (Worm wood) using leaf explants

Bhupendra Koul* and Owais Ahmad Lone

School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab (INDIA)-144411

ABSTRACT

Artemisia absinthium L. (worm wood) is an aromatic, perennial, medicinal and industrially important herb belonging to the family Asteraceae (compositae). The plants are distributed throughout the temperate climate of northern hemisphere of the world including Asia, Europe and North America. The aerial parts of the herb contains bioactive compounds like thujone, thujyl-alchol, cadinene, phellandrene, pinene etc. which possess a number of biological activities including anti-fungal, anti-spasmodic, anti-bacterial, anti-cancer, anti-oxidant, anti-rheumatic, anti-helminthic, analgesic, anti-inflammatory, anti-depressant, anti-parasitic, anti-septic, anti-malarial, anti-hepatotoxic, anti-viral, disinfectant and wound healing property. Nowadays, in vitro propagation is an essential tool of plant biotechnology by which endemic, endangered, medicinally and industrially important plants are propagated within short time. Due to the poor seed germination, A. absinthium may be propagated by tissue culture technique (in vitro culture). In the present study, the callus and shoot induction was optimized. For callus induction using leaf explant MS media supplemented with BAP (1.0 mg/l) + 2, 4-D (0.5 mg/l) was found to be optimal and for shoot induction MS media supplemented with BAP (3.5 mg/l) + 2, 4-D (0.5 mg/l) gave good response. The present study proposes a wide scope for multiple benefits of A. absinthum in the field of pharmaceuticals and many other industrial and economics ventures. A. absinthium has immense potential to act as panacea to several health related disorders and so its conservation through tissue culture before excessive exploitation is crucial.

Keywords: *Artemisia absinthium*, Worm wood, Anti-helminthic, *In vitro* regeneration, Callus induction medium.

INTRODUCTION

Ayurveda is the science of life (Ayur = Life, Veda = Science), which was developed in India thousands of years ago. The natural herbs have been used from ancient times for the treatment of various diseases and disorders [1]. In the developing countries, people use natural herbs for treating several diseases because they are safe and cost effective [2, 3]. Interest towards herbal drugs is increasing day by day which shall lead to limited access to modern medicines. It has been reported that more than sixty percent of the commercialized (approved) drugs are derived from plant sources [4]. Herbal formulations have replaced the allopathic medicines up to a greater extent [2, 3].

Artemisia genus of the family Asteraceae (compositae) is one such genus of herbal medicinal plant which has been traditionally used as a medicine from the past hundred years. Even today, *Artemisia* species are used in medicines for preparation of modern drug formulations. Besides the medicinal importance, *Artemisia* species also contain essential oils which are used for production of alcoholic beverages, cosmetics, insecticides and are as food additives. The bioactive compounds like 1,8-cineole, absinthin, artemisia ketone, camphene, phellandrene, pinene, thujene, terpinene and thujone possess strong action to fight diseases like hepatitis, cancer, diabetes, asthma, malaria, tuberculosis and fevers [5-10].

A. absinthium, a member of the genus *Artemisia* is an aromatic, endemic medicinal plant of Himalayas, and is revered for its numerous bioactive compounds, which have been traditionally used as medicines for the past

hundreds of years to treat several diseases. Most plants are aromatic and bitter in taste due to the presence of terpenoids and sesquiterpene lactones which provide the plants selective defense against biotic and abiotic stresses. Artemisia ketone, camphene, cadinene, phellandrene, pinene, terpinene, thujone, thujene, thujyl-alchol and 1,8-cineole are the major essential oils that were extracted from aerial parts of plant and possess numerous biological activities including anti-fungal [12, 12], anti-spasmodic, anti-bacterial, anti-hyperlipidemic [13], anti-cancer [14], anti-rheumatic, anti-oxidant, anti-microbial [7, 15, 12, 16], anti-helminthic, analgesic, anti-inflammatory [5], anti-depressant, anti-parasitic, anti-septic, anti-malarial, anti-hepatotoxic [17], anti-viral agents [5], disinfectant, and acaricidal [18]. *A. absinthium* also possess wound healing, [19], anti-snake venom [20], anti-leishmanial [21] activities. Crude plant parts of *A. absinthium* like leaves and stem are used to treat back pain, insect and spider bites [22], jaundice [23], labor pains, parasitic worm infections, skin wounds, stomach ailments [22], menstrual pains and used as a brain tonic [23]. Besides pharmacology, the *A. absinthum* is preferred for the manufacturing of alcoholic beverages, called absinthe, the distilled herbal spirit [24].

In a nut-shell, this plant has great relevance for human healthcare and so it's *in vitro* regeneration is important for maintaining the germplasm as well as to provide a continuous supply of raw material to the concerned pharmaceutical industries. Once a robust tissue culture protocol is established beneficial genes could also be incorporated by using *Agrobacterium*-mediated transformation technique which will protect the plant from diseases and may also help in enhancement of secondary metabolites.

Phytography

A. absinthium grows in humid soils or near water bodies in rocky areas with the sandy clay soil. The herb commonly grows in natural habitat on uncultivated-ground, rocky slopes, sides of footpaths, foot of hills, fields, pavements, roadsides and waste areas [13]. These are mainly distributed in temperate climates of northern hemisphere between mid to high latitudes. These plants grow in the North America (US and Canada), Europ, Afghanistan Siberia, India (Kashmir), Pakistan, Russia, Iran, china, Kazakstan, Japan, Mediterranean region and North Africa [5, 17, 25, 26, 27, 28, 29].

A. absinthium is a perennial, shrubby herb which is silver green in colour with hairy stem and bears yellow flowers (Figure 1). Stem is erect, grows to a height of 0.8–1.2 m, branched, leafy and woody at the base [30]. Petioles of leaves are 6 to 12 cm long with ovate to elliptic leaf blade. The leaves are of two kinds i.e. radical and stem leaves with bi- or tri-pinnatifid margin. Leaves possess aromatic and acrid taste due to the presence of terpenoids, glucosides (absinthin), sesquiterpenes (anabsinthin) and lactones. Root system is fibrous which also possess aromatic bitter taste. The flowering period is between June to September [5, 31, 32, 33]. The chromosome number of *A. absinthium* is 18 and the basic chromosome number is 9. [34].

Figure 1 *Artemisia absinthium*: A potted plant

Traditional uses

A. absinthium L. is commonly known by several names like 'Absinthium', 'Worm wood', 'Green ginger', 'Absinthe worm wood', 'Common worm wood' or 'Grand wormwood', 'Louisiana Artemisia', 'Cudweed', 'Western Mugwort' and ' White Sage'.The dried parts of plant are used as insecticide to protect the clothes against insect-damage. The whole herb is used for the preparation of different tonics for the betterment of health. Dried leaf powder is used to cure intestinal and gastric problem. Seeds are used in powdered form to treat rheumatism and seed paste is used to treat tooth-ache [26].The leaves of *A. absinthium* are soaked in water for overnight and the water-extract is consumed to reduce the labor pain. Due to the bitter taste of leaves, women apply it on their nipples to encourage the weaning of their babies [27]. The dried leaf powder is taken with honey to treat stomach disorders [35]. The young herbs are used to treat malaria while leaves are applied to wounds and chewed as anti-hypertensive. Infusion, a drink made by allowing something (such as tea) to stay in liquid (such as hot water) of whole herb is used to treat gastrointestinal ailments, stomachache, hypertension, digestive disorders and stomach-worms in children [28, 36].

Table 1: Summarized biological activities of *A. absinthium*

Pharmacological activities	Tested organism	Study model / methodology	Ref.(s)
Anti-microbial	*Staphylococcus aureus*	Rat Model	[12]
	Bacillus subtilis, Candida albicans Escherichia coli, Pseudomonas aeruginosa, S.aureus, Salmonella typhimurium, Saccharomyces cerevisiae and Enterecoccus faecalis	Disk diffusion method (DDM)	[16]
	E. coli	DDM	[7]
	B. subtilis, B. cereus Haemophilus influenza, Klebsiella pneumonia, P. aeruginosa and S. aureus	DDM	[41]
	Aspergillus niger, Alcaligenes faecalis, *Acinetobacter lwoffi, B. subtilis, B.cereus, C. albicans, E.coli, S. aureus, S. cereviciae, Streptococcus pyogenes, S. thermophilus, S. hominis, Salmonella typmiruim, P.aeruginosa, P. pseudoalcaligenes, P. fluorescens, P. putida, K. pneumonia, K. pneumonia, Enterobacter cloaceae, Proteus mirabilis, Providenciaal caliaciens, Flavobacterium indologenes, Penicillium brevicompactum, Trichothecium roseum and Yersinia enterocolitica*	DDM	[42]
Anti-bacterial	*E. coli and S. aureus*	Paper DDM	[43]
Anti-fungal	*C. albicans*	DDM	[37]
	Microsporum canis and C. albicans	DDM	[43]
Anti-oxidant	ABTS and ethanol extraction	ABTS radical cation (ABTS+)	[16]
	DPPH, Methanolic solution of DPPH (1 mM, 0.5 ml) + the samples. The spectrophotometer absorbance were measured at 517 nm.	DPPH	[7]
	Methanol extracts of *Artemisia absinthium*	*β*-carotene bleaching method	[42]
Anti-parasitic	*Toxocara cati*	Cats	[44]
Hypolipidemic	Rabbits, tissue lipids profile and serum lipid profile	Rabbits	[13]
Cytotoxicity (cytotoxic action on Human tumor cell line)	Human lymphocyte and Hela cells	MTT assay	[45]
	Human cell lines (adenocarcinoma and squamous non-small cell lung cancer, colorectal carcinoma, luminal breast adenocarcinoma, melanoma and bone marrow stromal)	WST-1 assay	[34]
Acaricidal activity	*Rhipicephalus sanguineus*	Egg hatchability test (EHT), Larval packet test (LPT) and Adult immersion test (AIT)	[18]
Anti-viral. (Anti-HIV-1)	Blood and a single-cycle HIV-1 Stock	MTT assay	[46]

Phytochemistry

Nowadays, HPLC and HPTLC has become regular analytical techniques due to their efficiency in quantitation of analytes at micro or even nanogram levels and cost effectiveness. Phytochemical analysis of *A. absinthium* has shown the presence of a range of unique compounds with several medicinal, nutraceutical and pharmaceutical properties as reported in table 1. The compounds reported are Artemisia ketone, Aromadendrene, Bicyclogermacrene, Borneol, Camphene, Carvacrol, Carvone, Caryophyllene oxide, Chamazulene,Chrysanthenyl acetate, *cis*-chrysanthenol, *cis*-epoxyocimene, *cis*-piperitone epoxide, *cis*-sabinol, *cis*-verbenol, Cubenol, Curcumene, Decanoic acid, Dihydro dodecanal, Dihydro chamazulene, (E)-anethole, (E)-crysantenyl acetate, (E)-linalool oxide, (E)-nerolidol, (E)-nuciferolisobutyrate, (E)-sabinyl acetate, (E)-sabinene hydrate, (E)-α-cadinol,(E)-ß-caryophyllene, (E)-ß-ocimene, (E)-3-hexenyl butyrate, Farnesol, Germacrene B, Germacrene D, Geranial, Geraniol , Geranyl acetate, Geranyl butanoate, Geranyl isobutanoate, Geranyl isovalerate, Geranyl isovaleriate, Geranyl propionate, Geranyl valeriate, Geranyl, 2-methylbutanoate, Iso-3-thujanol, Lavandulyl acetate, Lavandulylisobutanoate, Lavandulyl 2-methylbutyrate, Ledol, Limonene, Linalyl acetate, Linalool, Linalool acetate, Myrcene, Myrtenal, Myrtenol, *n*-heneicosane, *n*-heptadecane, *n*-nonadecane, Nerol, Neral, Neryl acetate, Neryl butanoate, Neryl isobutanoate, Neryl isovalerate, Neryl 2-methylbutanoate, Neryl-3-methylbutanoate, Neryl propionate, Neryl valeriate, *p*-cymene, Perillaldehyde, Piperitenone oxide , Pogostol, Pulegone, Sabinene, Santolina alcohol, Spathylenol, *trans*-caryophyllene, *trans*-Epoxyocimene, *trans*-pinocarveal, *trans*-Sabinol, *trans*-

Verbenol, Terpinen-4-ol,Terpinolene, Terpinyl propionate, Thymol, Tricyclene, *trans*-Muurolol , Valerenic acid, Verbenone, Viridiflorol, (Z)-anethole, (Z)-α-cadinol, (Z)-crysantenyl acetate, (Z)-linalool oxide, (Z)-nuciferolisobutyrate, (Z)-ß-Ocimene, (Z)-sabinyl acetate, α-copaene, α-bisabolol, α-dehydro-ar-himachalene, α-humulene, α-ionone, α-muurolene, α-phellandrene, α- pinene, α-santano, α-terpinene, α-terpineol, α-terpinyl acetate, α-thujene, α-thujone, β- bourbonene, β-citronellol, β-elemene, β-ionone, β-pinene, β-selinene,β-thujone, γ-muurolene, γ-cadinene , γ-dehydro-Ar-himachalene, γ-isogeraniol, γ-terpinene, δ-cadinene, 1,8-cineole, 3,6-dihydrochamazulene, 9-geranyl-p-cymene and , (-)-(5Z)-2,6-dimethylocta-5,7-diene-2,3-diol [5, 7, 16, 28, 32, 34, 37, 38, 39, 40].

EXPERIMENTAL SECTION

Plant Material

A. absinthium plants were collected from district Pulwama, (Jammu and Kashmir) and were identified by Dr. Arbeen Ahmad Bhat, Assistant Professor (Taxonomy), Lovely Professional University Punjab. Leaves were used as explants for *in vitro* shoot induction via intermediary callus phase.

Media for explants regeneration

In the tissue culture of *A. absinthium*, Murashige and Skoog medium (1962) [47], B_5 vitamins [48] supplemented with different growth regulators were used. The macronutrients solutions were prepared in 10X concentration. The micronutrients, iron-EDTA and B5 vitamins were 100X concentrated and prepared in double distilled water and stored at 4˚C. Different media used in the present study are shown in table 2.

Table 2: Different media used in the study

Media	Composition
Callus induction media CIM-A	MS + Maltose (3.0 %) + Agar (0.8 %) + BAP (0.5 mg/l) + IAA (0.5 mg/l)
Callus induction media CIM-B	MS + Maltose (3.0 %) + Agar (0.8 %) BAP (1.0 mg/l) + 2, 4-D (0.5 mg/l)
Shoot induction media SIM-A	MS + Maltose (3.0 %) + Agar (0.8 %) + BAP (3.5 mg/l) + 2, 4-D (0.5 mg/l)
Shoot induction media SIM-B	MS + Maltose (3.0 %) + Agar (0.8 %) + BAP (1.5 mg/l) + IAA (0.5 mg/l)

Surface sterilization of leaf explant

The leaf explants were initially washed with tap water for 8-10 min. Thereafter, the explants were washed by 5 % Tween-20 and rinsed 4-5 times with double distilled water. Then, explants were disinfected by 0.1 % mercuric chloride for 4-5 min followed by 4-5 rinses with sterilized distilled water. The explants were then treated with 75% (v/v) ethanol for 1.5 min followed by 5 rinses in sterilized distilled water. Then these explants were treated with 0.5% sodium hypochlorite for 3 min followed by 5 rinses with sterilized distilled water.

Inoculation of leaf discs on callus induction media

The leaf explant which was surface sterilized were dried on sterile blotting sheets with the help of sterilized forceps under the laminar air-flow and placed on semi-solid callus induction media, CIM- A [MS + Maltose (3.0 %) + Agar (0.8 %) + BAP (0.5 mg/l) + IAA (0.5 mg/l)] and CIM-B [MS + Maltose (3.0 %) + Agar (0.8 %) BAP (1.0 mg/l) + 2, 4-D (0.5 mg/l)], respectively for callus induction and incubated at 20 ± 2 ^{o}C in a culture room with 16 h photoperiod of light intensity 50 μmol m^{-2} s^{-1}photon flux density (PFD) and 60 % relative humidity (RH).

Shoot induction from leaf derived callus

The callus derived from leaf explants were transferred to shoot induction media, SIM-A [(MS + Maltose (3.0 %) + Agar (0.8 %) + BAP (3.5 mg/l) + 2, 4-D (0.5 mg/l)] and SIM-B [(MS + Maltose (3.0%) + Agar 0.8 % + BAP (1.5 mg/l) + IAA (0.5 mg/l)], respectively.

RESULTS AND DISCUSSION

Callus induction

The initiation of callus was observed after 15 days in CIM-A and 10 days in CIM-B. The responding percentage of callus initiation is show in table 3. Of the two media used for callus induction, CIM- A [MS + Maltose (3 %) + Agar (0.8%) + BAP (0.5 mg/l) + IAA (0.5 mg/l) and CIM-B [MS + Maltose (3.0%) + Agar (0.8%) BAP (1.0 mg/l) + 2, 4-D (0.5 mg/l)], the CIM-B media was found to be optimal as represented by a high percentage responding frequency of 90%. The callus induction response using both the media is shown in Figure 2.

Table 3: Percentage response of *A. absinthium* leaf explant for callus induction

Media	BAP (mg/l)	IAA (mg/l)	2,4-D (mg/l)	No. of explants inoculated	No. of responding explants	Responding explants (%)
CIM-A	0.5	0.5	-	20	9	45
CIM-B	1.0	-	0.5	20	18	90

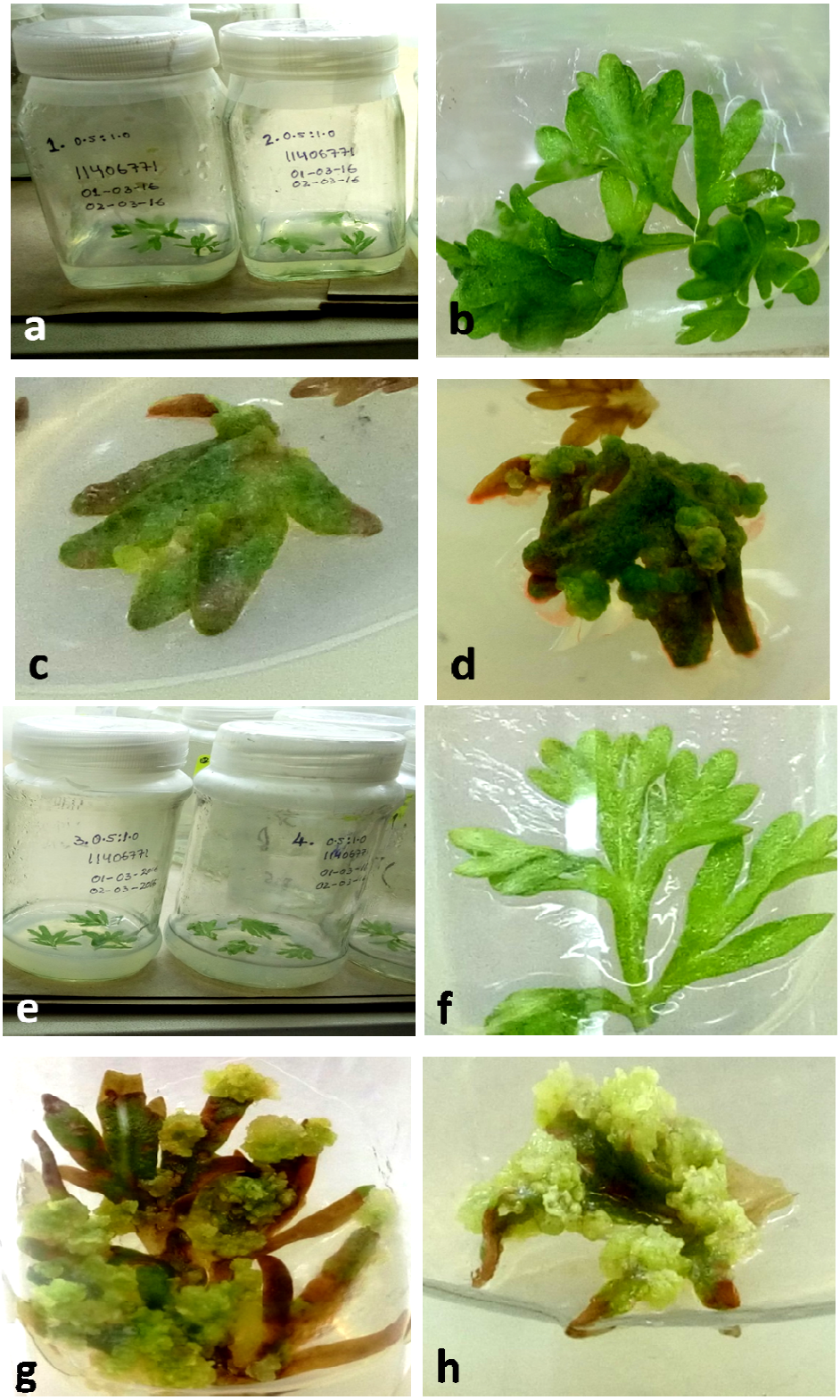

Figure 2 Callus induction on *A. absinthium* from leaf explant. [a, b] Leaf explant placed on CIM (A) [c, d] Few calli formed. [e, f] Leaf explant placed on CIM (B) [g, h] Vigorous callus growth

Shoot induction

For the shoot induction individual callus was inoculated on the shoot induction media SIM-A and SIM-B. The SIM-A contains MS media with 0.8 % agar supplement with BAP (3.5 mg/l) and 2, 4-D (0.5 mg/l) and SIM-B contains MS media with 0.8% agar supplement with BAP (1.5 mg/l) and IAA (0.5 mg/l). The percentage response of shoot buds is shown in table 4. The shoots that were developed on shoot induction media A (SIM-A) were transferred to shoot elongation media (SEM) containing MS salts plus GA3 (1.0 mg/l) followed by rooting on root induction media (RIM) containing MS salts plus IBA (0.5 mg/l).

Table 4: Shoot induction from callus

Medium	BAP (mg/l)	IAA (mg/l)	2,4-D (mg/l)	No. of explants inoculated	No. of responding explants	Responding explants (%)
SIM-A	3.5	-	0.5	10	8	80
SIM-B	1.5	0.5	-	10	6	60

Figure 3 Shoots developed on shoot induction media A (SIM-A)

Nowadays, *in vitro* propagation has become an essential tool of biotechnology by which endemic, endangered, medicinally and industrial plants are propagated within a short period of time. In vitro regeneration plays an important role in large scale multiplication of the plants, production of disease-free plants, induction of somaclonal variation etc. Transgenic technology can also be used for improving the secondary metabolite content by incorporating beneficial genes into the plant genome [49].The current feature of plant biotechnology is production of a large number of secondary plant products by using cell suspension culture, genetic engineering in order to produce GM crops and production of pharmaceutically important molecules from plants, such as vaccines and antibodies (molecular farming) [50, 51].

The *in vitro* regeneration of any plant species depends upon the optimum conditions of culture room, explants used, media composition, pH of the media, PGR's type and concentration. Proper sterilization of explants is an important parameter to avoid contamination. Plant cells possess totipotency which helps them to retain their meristematic state and can develop undifferentiated mass of cells known as callus from which whole plant can be generated (redifferentiation) [52, 53]. Totipotency of cells varies from tissue to tissue in plants, which could be promoted by using plant growth regulators so as to generate new plants either through direct organogenesis or somatic embryogenesis [52].

In the present study we have used two different media combinations (**CIM-A** and **CIM- B**), out of which **CIM-B** media [MS + Maltose (3.0%) + Agar (0.8%) + BAP (1.0 mg/l) + IAA (0.5 mg/l)] was found to be most optimal for callus induction. The **SIM-A** media [MS + Maltose (3 %) + Agar (0.8 %) + BAP (3.5 mg/l) + 2, 4-D (0.5 mg/l)] was found to be suitable for shoot induction from leaf explant derived callus. Meticulous pharmacological and phytochemical and tissue culture studies (especially suspension culture studies) on *A. absinthium* could yield reliable compounds of pharmacological relevance for better healthcare.

Abbreviations

BAP: benzyladenine; *CIM: Callus induction medium*; *DDM: Disk diffusion method*; *GA3: Gibberellic acid; h: Hour*; *HPLC: High Performance Liquid Chromatography*; *HPTLC: High performance thin layer chromatography*; *IAA: Indole-3-acetic acid*; *IBA: Indole butyric acid*; *mg: Milligrams*; *min: Minutes*; *PFD: Photon flux density*; *RH:*

Relative humidity; *RIM: Root induction media*; *SEM: Shoot elongation media*; *SIM: Shoot induction media*; *IAA: indole-3-acetic acid*; *MS: Murashige and Skoog*; *2, 4-D: 2,4-dichlorophenoxyacetic acid.*

Acknowledgements
The author is thankful to Mrs. Rashmi Mittal, Pro-Chancellor, Lovely Professional University (LPU), Punjab, India for her valuable suggestions and infrastructural support.

REFERENCES

[1] J Boik. Natural Compounds in Cancer Therapy. Oregon Medical Press, LLC, Princeton, Minnesota 55371, USA, **1996**. 1-503.
[2]RHO Bannerman; J Burton; WC Ch'en. **1983**. Geneva, World Health Organization
[3]NR Farnsworth; O Akerele; AS Bingel; DD Soejarto; Z Guo. *World Health Org.*, **1985**, 63, 965-981.
[4]GM Cragg; DJ Newman. *Biochim. Biophys. Acta.*, **2013**, 1830(6), 3670-3695.
[5]MJ Abad; LM Bedoya; L Apaza; P Bermejo. The *Artemisia* L. Genus, A Review of Bioactive Essential Oils. *Molecules* **2012**, 17, 2542-2566.
[6] M Aziz; A Karim; E-M El-Ouariachi; A Bouyanzer; S Amrani; H Mekhfi; A Ziyya; A Melhaou; M Bnouham; A Legssyer. *Sci. Pharm.* **2012**, 80, 457-467.
[7] S Erel; G Reznicek; SG Senol; NÜK Yavasogulu; S Konyalioglu; AU Zeybek. *Turk. J. Biol.*, **2012**, 36, 75-84.
[8] K Younes; S Merghache; N Djabou; D Merghache; A Muselli; B Tabti; J Costa. *Afr. J. Pharm. Pharmacol.* **2012**, 6(42), 2912-2921.
[9] H Ben-Nasr; MAB Abderrahim; M Salama; K Ksouda; K Zeghal. *J. App. Pharm. Sci.*, **2013**, 3(5), **120**-125.
[10]TR Rashmi; MS Francis; S Mural. *J. Pharmacogn. Phytochem.*, **2014**, 3(4), 160-163.
[11] K Saban; M Recep; CAA Ahmet; Y Ali. *J. Agric. Food Chem.*, **2005**, 53, 9452-9458
[12]HR Moslemi; H Hoseinzadeh; MA Badouei; K Kafshdouzan; RM Nezhad-Fard. *Indian J. Microbiol.*, **2012**, 52(4), 601-604
[13]HM Daradka; M Badawneh; JA Al-jamal; Y Bataineh. *World Appl. Sci. J.*, **2014**, 31(8), 1415-1421.
[14]G Shafi; TN Hasan; NA Syed; AA Al-Hazzani; AA Alshatwi; A Jyothi; A Munshi. *Mol. Biol. Rep.*, **012**, 39(7), 7373-7379.
[15]F Juteau, I Jerkovic, V Masotti, M Milos, J Mastelic, JM Bessière, J Viano. *Planta Med*., **2003**, 69, 158-161.
[16]A Altunkaya; B Yıldırım; K Ekici; O Terzioglu. *GIDA/ The Journal of Food*, **2014**, 39 (1), 17-24.
[17]KS Bora; A Sharma. *J. Pharm. Res.*, **2010**, 3, 325-328.
[18]R Godara; S Parveen; R Katoch; A Yada; PK Verma; M Katoch; D Kaur; A Ganai; P Raghuvanshi; NK Singh. *Parasitol. Res.*, **2014**, 113,747-754.
[19]B Kour; G Kour; S Kaul; MK Dhar. *Advances in Botany*, **2014**, Volume, Art (ID 727020), 7.
[20]A Nalbantsoy, SB Erel, C Koksal, B Gocmen, MZ Yildiz, NU Karabay Yavasoglu. *Toxicon*, 2013, 65, 34-40.
[21]S Khanjani Jafroodi; A Farazmand; M Amin; A Doroodgar; MR Shirzadi; MR Razavi. *International Archives of Health sciences*. **2015**, 2(2), 69-74.
[22]PM Guarrera. *Fitoterapia*, **2005**, 76, 1-25.
[23]MS Shekhawat; M Manokari. *Chin. J. Biol.*, **2015**, 2015, Article ID 273405, 8 pages.
[24] Jiří Patočka; Bohumil Plucar. *J. Appl. Biomed.*, **2003**, 1, 199-205.
[25]J Valles; ED McArthur. "Artemisia systematics and phylogeny, cytogenetic and molecular insights," in Proceedings of the Shrubland Ecosystem Genetics and Biodiversity, E. D. McArthur and D. J. Fairbanks, Eds., pp. 67-74, UT Ogden, US Department of Agriculture Forest Service, Rocky Mountain Research Station, Provo, Utah, USA, **2001**.
[26]MQ Hayat; MA Khan; M Ashraf; S Jabeen. *Ethnobotany Research & Applications*, **2009**, 7, 147-162.
[27]DW Lachenmeier. *J. Ethnopharmacol.*, **2010**,131, 224-227.
[28]FS Sharopov; VA Sulaimonova; WN Setze *Rec. Nat. Prod.*, **2012**, 6(2), 127-134.
[29] AG Pirbalouti; M Firoznezhad; L Craker; M Akbarzadeh. *Rev. Bras. Farmacogn.*, **2013**. 23, 861-869.
[30] PK Ashok; K Upadhyaya. *J. Pharmacogn. Phytochem.*, **2013**, 1(6), 229-235.
[31] A Razaeinodehi; S Khangholi. *Pak. J. Biol. Sci.*, **2008**, 11(6), 946-949.
[32]BJ Goud; V Dwarakanath; BKC Swamy. *International Journal of Advanced Research in Engineering and Applied Sciences*, **2015**, 4(5), 78-107.
[33] RA Martínez–Díaz; A Ibáñez–Escribano; J Burillo; LdeL Heras; GD Prado; MT Agulló–Ortuño; LF Julio; A González–Coloma. *MemInst Oswaldo Cruz*, **2015**, 110(5), 693-699.
[34]K Konowalik; S Garcia; J Pellicer; A Kreitschitz; J Valles. *Ann. Bot. Fennici.*, **2010**, 47, 477-488.
[35] E d'Avigdor; H Wohlmuth; Z Asfaw; T Awas. *J. Ethnobiol. Ethnomed*., **2014**, 10, 38
[36]KA Tariq; MZ Chishti; F Ahmad; AS Shawl. *Vet. Parasitol.*, **2009**, 160(1-2), 83-88.
[37]D Obistioiu; RT Cristina; I Schmerold; R Chizzola; K Stolze; I Nichita; V Chiurciu. *Chem. Cent. J.*, **2014**, 8(6), 1-11.

[38] H Wani; SA Shah; JA Banday. *India J. Phyto*., **2014**, 3(2), 90-94.
[39]G Özek; Y Suleimen; N Tabanca; R Doudkin; PG Gorovoy; F Göger; DE Wedge; A Ali; IA Khan; KHC Başer. *Russia. Rec. Nat. Prod.*, **2014**, 8(3), 242-261.
[40] A Orav; A Raal; E Arak; M Müürisepp; T Kailas. *Proc. Estonian Acad. Sci. Chem.*, **2006**, 55(3), 155-165.
[41] R Habibipour; M Rajabi. *J. HerbMed Pharmacol.*, **2015**, 4(4), 133-137.
[42] M Sengul; S Ercisli; H Yildiz; N Gungor; A Kavaz; B Çetina. *Iran. J. Pharm. Res.*, **2011**, 10(1), 49-56.
[43] AFC Valdes; JM Martinez; RS Lizama; M Vermeersch; P Cos; L Maes. *Mem. Inst. Oswaldo. Cruz.*, **2008**, 103(6), 615–618.
[44] K Yildiz; M Basalan; O Duru; S Gokpinar. *T¨urkiye Parazitoloji Dergisi*, **2011**, 35 (1), pp. 10-14.
[45] M Taherkhani. *Iran. J. Toxi.*, **2014**, 8(26), 1152-1156.
[46] H Mohabatkar; M Behbahani, MRR Nejad. *J. Coast. Life Med.*, **2015**, 3(12), 996-999.
[47] T Murashige; F Skoog. *Physiol. Plant.*, **1962**, 15(3), 473-497.
[48]OL Gamborg; RA Miller; K Ojima. *Exp. Cell. Res.*, **1968**, 50(1), 151-158.
[49]B Koul; S Srivastava; DV Amla; I Sanyal. *Int. J. Biosci.*, **2014**, 4, 51-69.
[50] E Ferrante; D Simpson. *Biological & Biomedical Sciences*, **2001**, 4(1).
[51]P Christou; T Capell; A Kohli; JA Gatehouse; AMR Gatehouse. *Trends Plant Sci.*, **2006**, 11, 302-308.
[52]SS Bhojwani; MK Rajdan. Plant tissue culture: theory and Practice, a revised Edition, Volume 5, Elsevier Publication, Netherland, **1996**, 1-767.
[53] EF George; MA Hall; G-JDe Klerk. Plant propagation by tissue culture. Vol 1, 3rd edition, Springer Netherlands. **2008**.