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Appendices

Appendix 1: Preparation of stock solutions for MS (Murashige & Skoog) medium

(Murashige and Skoog, 1962)

Stock solutions	Ingredients	Composition in media (mg/ L)	Stock Solution (w/v) (g)	Volume in media
MS Macro I (10 X) 1000ml	NH4NO3	1650	16.5	100ml
	KNO3	1900	19	
	MgSO4.7H2O	370.6	3.7	
	KH2PO4	170	1.7	
MS Macro II (10 X) 1000ml	CaCl2.2H2O	439.8	4.398	100ml
Fe-Na EDTA (1000 X) 100ml	Fe-Na EDTA	36.7	36.7	1ml
Micro Nutrients (1000 X) 100ml	NaMoO4.7H2O	0.2	0.025	1ml
	CuSO4.5H2O	0.025	0.0025	
	CoCl2.2H2O	0.025	0.0025	
	MnSO4.4 H2O	13.2	1.32	
	ZnSO4.4H2O	8.6	0.86	
	H3BO3	6.2	0.62	
KI (1000X) 100ml	KI	0.83	0.083	1ml
MS Vitamins (1000 X) 100ml	Nicotinic Acid	0.5	0.05	1ml
	Pyridoxine HCl	0.5	0.05	
	Thiamine HCl	0.1	0.01	
Myo-Inositol				0.1g
Glycine 5ml	Glycine	2	0.2	1ml

Preparation of MS medium

1. To make 1 L of MS medium, the required volume of each stock solution, 100mL macronutrients I & II, 1 mL micronutrients, 1 mL iron source, 1mL potassium iodide and 1mL vitamins were added into a 2 L beaker containing 200-250 mL of distilled water and stirred magnetically.

2. Then, 15 g sucrose, 0.1 g myo-inositol and 1 mL of glycine (0.01 g glycine in 5 mL of distilled water) were added and stirred to achieve complete dissolution. Growth regulators i.e. auxins 0r cytokinins were added as per requirement.

3. The volume was made up to approximately 450 mL (950 mL for suspension media) with distilled water. The pH was adjusted to 5.70±0.1 with 1 N NaOH and/or HCl.

4. The whole of the content was transferred to a 500 mL (1 L for suspension) measuring cylinder and the volume was made up to the mark with distilled water.

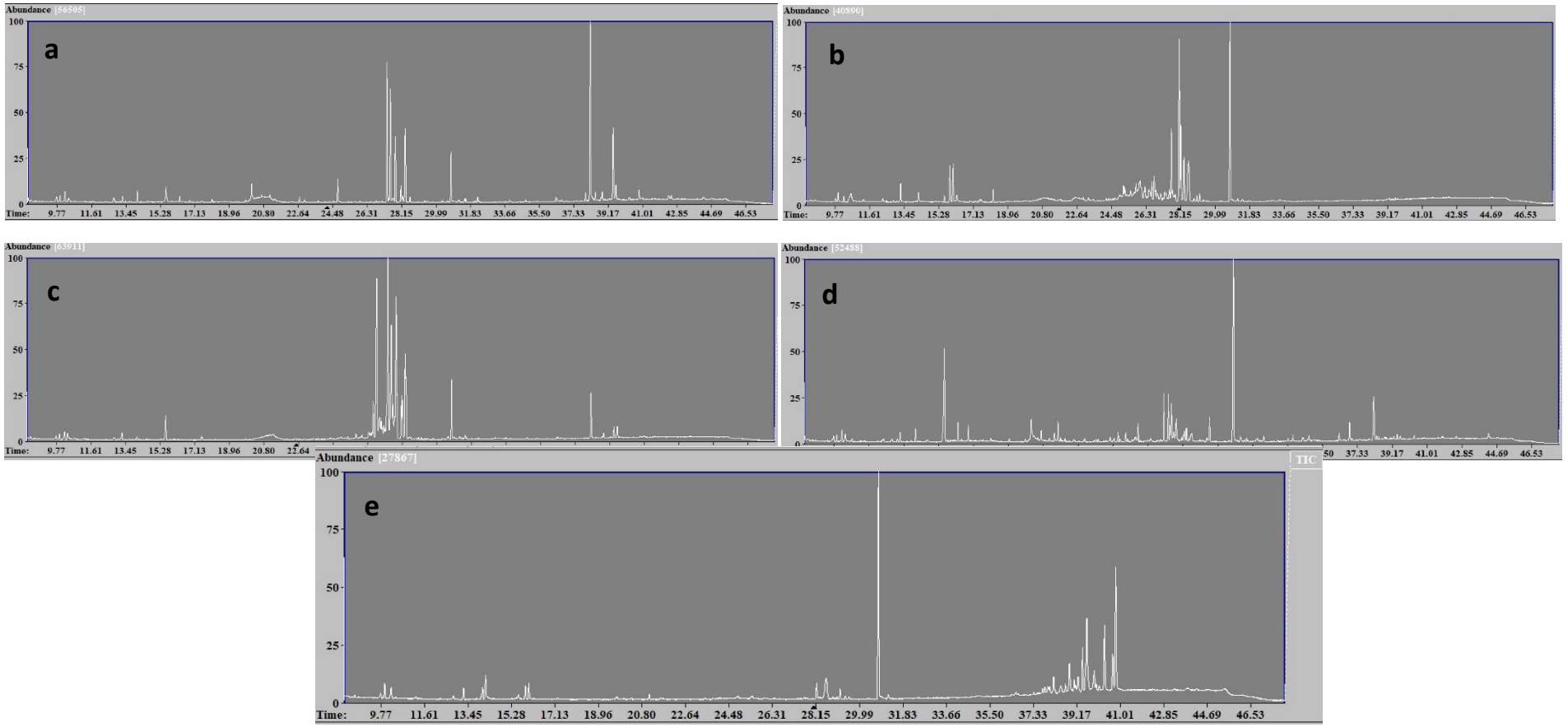
5. For MS agar medium, 8 g of agar was added to 500 mL distilled water and stirred for complete mixing. Agar was dissolved by heating the content in a microwave oven.

6. The medium and agar solution were mixed well.

7. The medium was transferred into Schott bottle (suspension media) and/or the medium was distributed (approximately 25-30 mL) to each sterile bottle (solid media). Bottles were labelled before autoclaving.

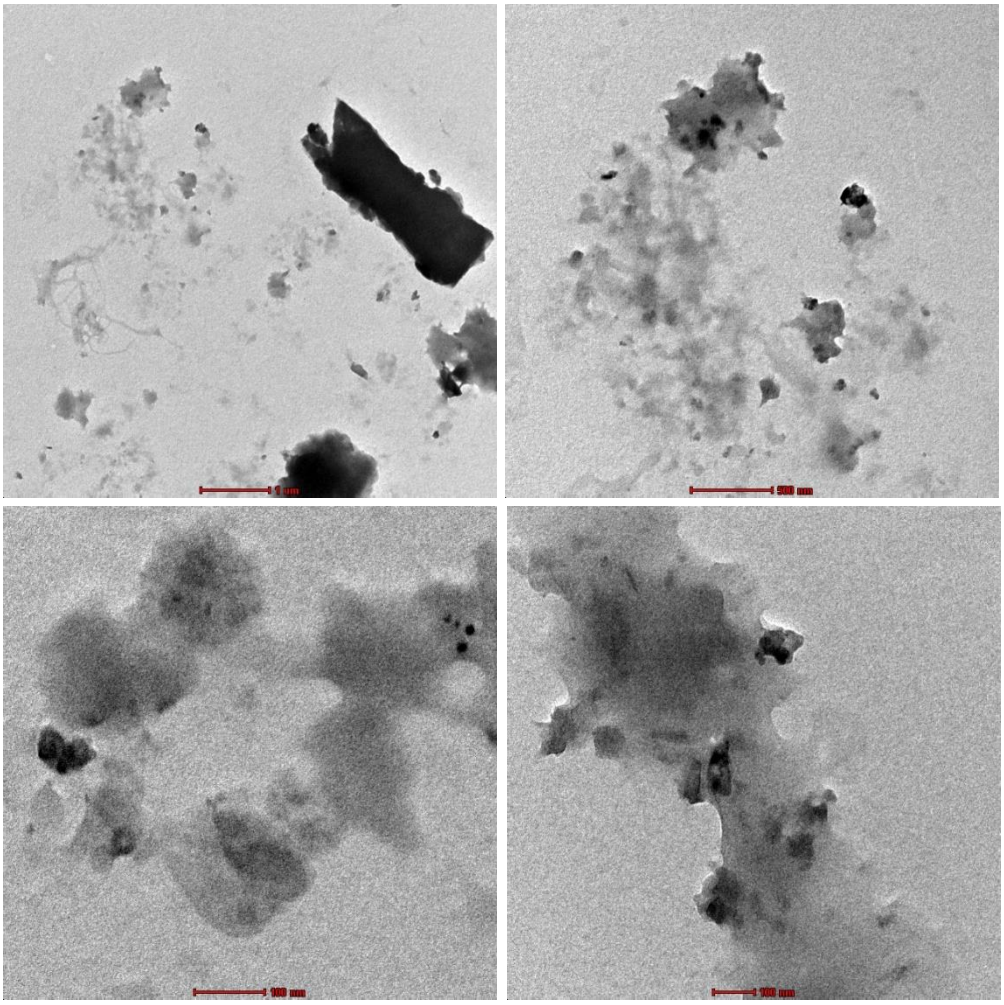
8. The culture medium was autoclaved for 15-20 min at 121℃ and 15 psi.

Appendix 2: GC-MS chromatogram of *W. somnifera* extracts



Samples: a) IVS; b) E1D3T6; c) E2D5T6; d) FGS; e) FGR

Appendix 3: TEM analysis on CT6 (*in vitro* control shoot) of *W. somnifera*



Appendix 4: Collection of mitochondrial complex I protein subunits

Uniprot ID	Subunit/gene name	Protein name	PDB chain name	Sequence Length	Complex I module
P28331	NDUFS1	NADH-ubiquinone oxidoreductase 75kDa subunit, mitochondrial	L [auth M]	687	N module
P49821	NDUFV1	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	A	431	N module
P19404	NDUFV2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	N [auth O]	212	N module
O75306	NDUFS2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	P [auth Q]	430	Q module
O75251	NDUFS7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	C	156	Q module
P03886	ND1	NADH-ubiquinone oxidoreductase chain 1	PA [auth s]	318	Q module
P03905	ND4	NADH-ubiquinone oxidoreductase chain 4	OA [auth r]	459	P module

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Coimbatore - 641 043, Tamil Nadu, India

Appendix L2

(Item No 5 of Check List)
Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	Influence and distribution of lead nitrate on growth and secondary metabolite accumulation in *Withania somnifera* (L.) Dunal *in vitro* shoots	Journal of Phytology	15/179-186/2023	Scopus Indexed
2	Comparative adaptogenic properties of *Withania somnifera* and *Panax ginseng*	Current Botany	13/012-18/2022	UGC-CARE

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : N. Kanimozhi

Supervisor : 1/4/2024

HoD

Checked By:

1/4/2024
Dean of Respective School

The scholar Miss. Kanimozhi, N has published her articles in the following journals:

1. Journal of Phytology - indexed and active in Scopus from 2019 to present and
2. Current Botany - indexed and active in UGC Care Group I from June 2019 to present.

This may be considered.

01.04.24

Current Botany 2022, 13: 012-18
doi: 10.25081/cb.2022.v13.7599
https://updatepublishing.com/journal/index.php/cb

Review Article

ISSN: 2220-4822

Comparative adaptogenic properties of *Withania somnifera* and *Panax ginseng*

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ABSTRACT

Adaptogens are natural (herbs) or synthetic compounds (levamisole, aphobazole, etc) used to maintain stability in the human body. The plant based adaptogens were mainly used to enhance the physical endurance and mental health of patients. However, adaptogens are widely studied for their ability to protect and cope up the body against physical, chemical and biological stress and related diseases. *Panax ginseng* and *Withania somnifera* are natural adaptogens, used to attenuate stress & related disorders without increasing oxygen consumption. This review deals with a detailed description of the adaptogenic potential of *Panax ginseng* and *Withania somnifera* in improving human health. It also focuses on the similarity and mechanism of action of *Panax ginseng* and *Withania somnifera* as adaptogens on human stress induced disorders.

KEYWORDS: Adaptogen, *Withania somnifera*, *Panax ginseng*, stress

INTRODUCTION

Stress is a normal part of the modern lifestyle, which has a negative impact on one's mental and physical well-being. Acute stress is for a short duration due to work pressure, exertion, increased physical activities or similar things. Recent studies have revealed that traditional herbal medicines may offer promising alternatives for depression treatment with high safety and tolerance (Jin *et al.*, 2019). Chronic stress is the most serious among different stresses which may prolong for weeks, months or even for years. Chronic stress if left untreated leads to stress related defects such as hypertension, heart disease, anxiety, depression, memory impairment and chronic fatigue syndrome which is shown in Figure 1. (Provino, 2010; Salve *et al.*, 2019). Many natural herbs have been playing as an adaptogen in managing chronic stress and its related illness. Some of these herbs are *Withania somnifera*, *Panax ginseng*, *Eleutherococcus senticosus*, *Schisandra chinensis*, *Glycyrrhiza glabra*, *Rhodiola rosea*, *Bacopa monniera*, *Lepidium meyenii* and *Centella asiatica*. These adaptogens from plant extracts have been found to increase resistance against stress and stress related conditions (Khanum *et al.*, 2005; (Panossian & Wagner, 2005; Liao *et al.*, 2018; Todorova *et al.*, 2021). Plant adaptogens associated with human/animal studies have shown increased cortisol level, antioxidant capacity, mental and physical performance and prevented scopolamine induced oxidative stress (Giridharan *et al.*, 2011; Zarabi *et al.*, 2018).

ADAPTOGEN PROPERTY

An adaptogen is a compound that increases the resistance or normalizes the influence of multiple stressors without affecting normal body functions (Oliveira & Leitão, 2016). The term 'adaptogen' was coined by the Russian scientist, Lazarev, in 1947 while working on dibazol (2 benzyl-benzimidazole), a synthetic compound found to stimulate nonspecific resistance of organisms (Brekhman & Dardymov, 1969; Todorova *et al.*, 2021). The term Adaptogen was defined as "state of non-specific resistance" in stress (Brekhman & Dardymov, 1969; Panossian, 2003)a physiological condition that is linked with various disorders of the neuro endocrine–immune system (Stratakis & Chrousos, 1995). This definition has been updated as "*Adaptogenic substances have the capacity to normalize body functions and strengthen systems compromised by stress. They have a protective effect on health against a wide variety of environmental assaults and emotional conditions*" (Panossian *et al.*, 2021). Lazarev defined 'adaptogens' as agents which allow an organism to counteract any adverse physical, chemical or biological stressor by generating nonspecific resistance and thus becoming 'adapted' to diverse demands imposed on it (Lazarev *et al.*, 1959). Most of the adaptogens has antioxidant and anxiolytics property which attributes their adaptogenic property.The intake of adaptogens associated with affecting the hypothalamic pituitary adrenal axis and also affects cortisol and nitric oxide (NO) level.For instance, after consuming plant adaptogens, the level of cortisol and NO

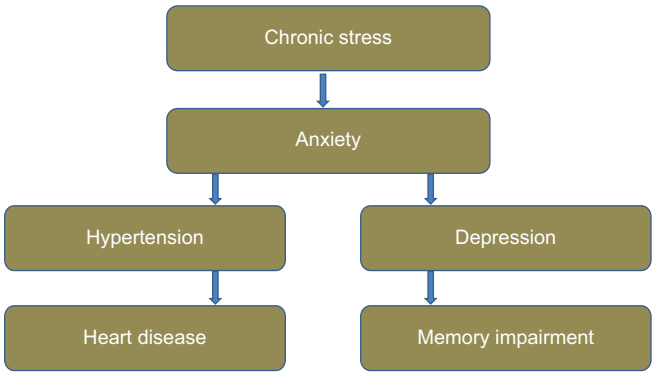

Figure 1: Chronic stress and its related defects

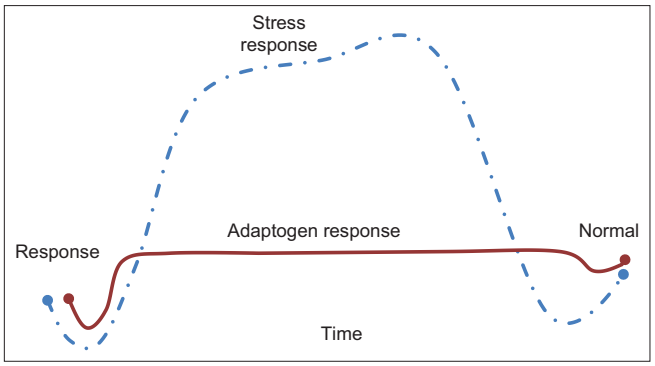

Figure 2: Effect of adaptogen on stress response

is not increased during physical exercise, but the adaptogens actually increases the level of messenger substance that increase NO stress decrease cortisol stress (Liao *et al.*, 2018). Effective use of these medicinal plants is associated with mental diseases and behavioral disorders, cognitive function and stress induced diseases. Adaptogen alters the disease course and maintains good health (Panossian *et al.*, 2021). Modern neutraceuticals has a wide range of herbal adaptogens under the labels of "Anti-ageing", "Antioxidants", "Immunity Boosters", "Anti-stress" formulas. Adaptogen effects on animals and cell lines exhibit neuroprotective, anti- fatigue, antidepressive, anxiolytic, nootropic, and CNS stimulating and tonic effects (Panossian & Wagner, 2005; Panossian & Wikman, 2010). The stress protective effect of adaptogens shown in Figure 2 is regulated by homeostasis via mechanisms such as stress-activated protein kinase c-Jun N-terminal protein kinase (JNK) (Panossian *et al.*, 2007), forkhead box O (FOXO) transcription factor (DAF-16) (Wiegant *et al.*, 2009), and molecular chaperones (Hsp70) (Panossian & Wikman, 2010), cortisol and nitric oxide (NO) (Chiu & Ko, 2004; Panossian & Wikman, 2010; Panossian *et al.*, 2012; Lopresti *et al.*, 2019). It was then believed that the main action of adaptogenic herbs was to up regulate and stress mimetic effects on the 'stress sensor' protein Hsp70 which helps in cell survival. Hsp70 affects nitric oxide and cortisol levels by inhibiting the expression of the NO synthase II gene and interacting with glucocorticoid receptors via JNK pathway. Prevention of increase NO and decrease in ATP due to stress results in increased endurance and performance. Thus up-regulation of Hsp70 with the help of adaptogen interacts with DAF-16 and JNK-1 mediated pathways, regulating the resistance to stress (Panossian *et al.*, 2009).

PANAX GINSENG AS AN ANTI-STRESSOR

Panax ginseng is the first clinically used adaptogen and has been extensively investigated clinically for its stress attenuating activity (Todorova *et al.*, 2021). *Panax ginseng* is used medicinally in most of the East Asian countries and is the most widely taken herbal product. Studies show that ginseng plays a significant role in depression and may act as potential antidepressant (Jin *et al.*, 2019). It is used as an alternative medicine with Anti-diabetic, anti-cancer, cardioprotective, neuroprotective and anti-inflammatory properties (Patel & Rauf, 2017). The active compound of the *Panax ginseng* is the ginsenosides (Saba *et al.*, 2018). One of the main indications for prescribing *Panax ginseng* is to improve the physical and mental status of an individual and to improve overall performance while under stress (Oliynyk & Oh, 2013). The Korean ginseng root has been reported to have a superior regulation of stress and is used to treat various ailments with its mysterious power (Choi, 2008; Lee & Rhee, 2017). It has actoprotective effect that improves physical and mental health while under stress (Oliynyk & Oh, 2013) and have been reported to have anti-stress effect (Tachikawa & Kudo, 2004; Lee & Rhee, 2017). The main constituents of *Panax ginseng* root consists of complex mixture of saponins known as ginsenosides of 38 types (Choi, 2008; Liao *et al.*, 2018).

The Korean red ginseng is produced by steaming which hydrolyses and converts ginsenosides into other types of ginsenosides such as Rb1, Re, Rf1, Rc, Rs3, Rs4, Rh2, Rh4, Rg1, Rg3, Rg5 which play an important role in the anti-oxidant and anti-stress activities (Nam 2005; Kim, 2018). Further, Ginsenosides contains active ingredients like Protopanaxadiols: Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2. Protopanaxatriols: Rg1, Rg2, Re, Rf, Rh1, Rh3 and oleanolic acid: Ro which has the following effects such as anti-cancer, anti-aging, immunomodulation, CNS regulation (Jin *et al.*, 2019). Although studies have been demonstrated in reduction of stress in animals after ginseng administration, description on the level of factors that alter the stress levels remain unclear. On a treadmill running test, administration of ginseng increased the endurance time to exhaustion, the basal level of ACTH and the corticosteroids (Filaretov *et al.*, 2009). Administration of ginseng also increased the superoxide dismutase, glutathione peroxidase and catalase levels and decreased malondialdehyde levels antagonistic to stress (Kim *et al.*, 2005; Sohn *et al.*, 2013). Ginsenoside Rg1 was found to decrease oxidative stress induced brain damage by the agent 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (Chen *et al.*, 2008). Ginseng extract administration also prevented oxidative damage to rat muscle in response to intense exercise (Voces *et al.*, 2004). Administration of Rg3 and Rb1 ginsenosides decreased the polyamine compound putrescine which is an potent stress indicator and Rb2, Rg1 decreased IL-6 in the plasma level (Kim *et al.*, 2003; Lee *et al.*, 2004). Ginsenoside Rb1 decreased the cell death due to 6-hydroxydopamine in neuroblastome cells (Hwang & Jeong, 2010) and protected dopaminergic cells from oxidative stress (Radad *et al.*, 2004; Kim *et al.*, 2008).

A comparative study was done on rats to investigate the anti-stress effect of *Ginkgo biloba* and *Panax ginseng*. In this the rats were subjected to acute and chronic stress and were treated with both the herbs. The animals were sacrificed at the end of the study to check stomach ulcer and vario0us other biochemical parameters such as creatine kinase, plasma glucose level, triglycerides, cholesterol and serum cortisterone. It was assessed that the *Panax ginseng* has the ability to treat chronic stress and its related effects whereas the *Ginkgo biloba* was able to treat only acute stress (Rai *et al.*, 2003). In another study, 90 males and females were divided into three groups and were exposed to cold environment stress. Then the three groups were allocated to receive *Panax ginseng* extract, placebo and a standard drug nifedipine which blocks calcium channels. The herb seems to be more effective than the drug in dilating the blood vessels followed by increase in the blood flow in the cold condition (Kaneko, 2004).

Ginsenosides attenuates excitotoxicity induced by kainic acid in hippocampal neurons. From this study, researchers suggest that the ginseng may have anticonvulsant activity (Shin *et al.*, 2009; Han *et al.*, 2012). Ginsenoside, Rg2 found to protects adrenal phaeochromocytoma cells from glutamate induced neurotoxicity (Li *et al.*, 2007), and hippocampal neural cells exposed to oxygen-glucose depleted state (Ye *et al.*, 2009). Ginseng also suppressed gastric ulcer formation against long term stress and effectively maintained creatine kinase, cholesterol, plasma glucose and triglyceride level after exposure to stress (Rai *et al.*, 2003; White *et al.*, 2016). Ginseng total saponins were found to protect against oxidative stress induced by cyclophosphamide in bone marrow cells, peripheral lymphocytes in mice and liver injuries in rats (Zhang *et al.*, 2008) through induction of cytochrome P450 expression and regulation of NO pathway in the liver of rats (Chen *et al.*, 2021).

Panax ginseng was studied in various *in vitro* models and was found that saponins rich fraction greatly reduced cortisol and catecholamine secretion from adrenal glands (Tachikawa & Kudo, 2004).People under a lot of stress usually have less REM and disturbed sleep. Fermented ginseng was found to improve an individual's sleep by anxiolytics effect via GABAergic modification (Kitaoka *et al.*, 2009). *Panax ginseng* stands at the top of all Chinese medicinal plant and has a great reputation as an adaptogen (Wyk & Wink, 2004).

WITHANIA SOMNIFERA AS A POTENT ANTI-STRESSOR

Withania somnifera (Ashwagandha) has been used for thousands of years as a popular remedy for many conditions. Perhaps its main use, as described in Ayurvedic literature, is as a "rasayana" or rejuvenating drug. The word Ashwagandha indicates the equine (of horses) odour of the plant. Another name Avarada suggests the application of this plant for enhancing longevity. Ashwagandha was found to act as a potent anti-oxidant and counteract stress to promote wellness (Singh *et al.*, 2011). Withania has the most significant adaptogenic effects which mainly results from the complex steroidal withanolides found in the roots (Braun & Cohen, 2015; White *et al.*, 2016). Traditionally, *Withania somnifera* has been used to stabilize mood in patients with behavioral disturbances. Researches revealed that Withania produces anti-anxiety and anti-depressant effects in rats which are very similar to the drugs such as Lorazepam for anti-anxiety, Imipramine for anti-depressant (Archana & Namasivayam, 1998; Upadhyay *et al.*, 2016).

Chronic stress affects immune functioning and increases the susceptibility to diseases (Kour *et al.*, 2009). Withania role in normal immune functioning against such kinds of stress was confirmed by a study conducted on albino mice to observe the effect of withanolide A from the root extracts of *Withania somnifera* on chronic stress induced alterations on cytokine secretion pattern and T lymphocyte subset distribution. It showed significant recovery of stress induced decrease in the T lymphocyte count resulting in the decreased corticosterone concentration and increased expression of IL-2 and IFN-gamma (Kour *et al.*, 2009). It was found that the Withania also has the ability to improve human's innate and adaptive immunity without any side effects by increasing the levels of immunoglobulins, cytokines and TBNK cells (Tharakan *et al.*, 2021). *Withania somnifera* have been found to protect against leukopenia (decreased WBC count) and bone marrow suppression caused due to cyclophosphamide. Administration of *Withania somnifera* root extract was also found to stimulate immunological activity in balb/c mice. Withania can also be used to treat memory loss due to chronic stress. The effect of withanolide A on memory deficient mice showed significant regeneration of axons and dendrites and also reconstruction of presynapses and postsynapses in the damaged neurons (Kuboyama *et al.*, 2005). Further, Withanolide A increases glutathione biosynthesis in rat neuronal cells by up regulating GCLC level through Nrf2 pathway and reduces neurodgeneration (Baitharu *et al.*, 2014).

Withania possesses an anti-stressor effect and is said to decrease stress induced changes.This antistress property of *Withania somnifera* have been investigated in a study of cold-water swimming stress test using adult Wistar strain albino rats. The results indicated that the drug treated animals show better stress tolerance (Gajarmal *et al.*, 2001). Ashwagandha's withanolides have been researched in a variety of animal studies examining their effect on numerous conditions, including immune function and even cancer (Ali *et al.*, 2001; White *et al.*, 2016). The extracts of seeds of *Withania somnifera* given to albino rats improved the protection against stomach ulcer caused due to stress (Singh *et al.*, 1982; White *et al.*, 2016). *Withania somnifera* suppressed stress induced gastric ulcer more effectively compared to the standard drug Ranitidine (Bhatnagar *et al.*, 2005). Stress induced stomach ulcer was hindered by pre-treatment with sitoindosides VII and VIII and the forced swimming stress decreased the duration of the immobility after giving sitoindosides VII and VIII (Ghosal *et al.*, 1989; Singh *et al.*, 2011).In a research the animals received a mild electric foot shock for a period of 21 days and the stress effects on animals produced glucose intolerance, hyperglycemia, gastric ulceration, immunosuppression, mental depression and

male sexual dysfunction (Bhattacharya & Muruganandam, 2003; Salve *et al.*, 2019). Animals treated with *Withania somnifera* everyday one hour before the foot shock treatment experienced a reduced level of stress and its effects. This confirms Ashwagandha as a significant anti-stress adaptogen (Bhattacharya *et al.*, 2001). Department of Pharmacology, University of Texas Health Science centre conducted research on Ashwagandha plant extracts and found that it produces GABA like activity which accounts for the herbs anti-stress effects (Mehta *et al.*, 1991). Gamma amino butyric acid (GABA) is an inhibitory neurotransmitter and decreases the neuron activity from over firing, thus producing a calming effect. Rats were protected from stress induced stomach ulcer and also showed improvement in both short range and long range memory after oral application of sitoindosides IX and X (Ghosal *et al.*, 1989). These results show that sitoindosides VII, VIII, IX and X represent adaptogenic substances of *Withania somnifera* (Singh *et al.*, 2011).

A study on the effect of chronic electric shock for 14 days showed a significant decrease in the nor-adrenaline (NA) and Dopamine (DA) levels and increased 5-hydroxytryptamine (5HT) in regions of brain (Bhattacharya *et al.*, 2002). EuMil is a polyherbal formulation which consist*Withania somnifera* as its key ingredient was administered on 14 days treatment and was found to normalize the level of NA, DA, 5HT induced by chronic stress (Bhattacharya *et al.*, 2002). Treatment with *Withania somnifera* for two weeks was found to reverse both Ibotenicacids (IA) induced cognitive deficit and the reduction of cholinergic markers thus promoting the learning and memory capacity. In a human clinical trial of 20 patients suffering from anxiety disorder, the anxiolytics potential of *Withania somnifera* has been significantly evaluated (Andrade *et al.*, 2000). Since anxiety is the outcome of chronic stress, Withania has a role in stress management (White *et al.*, 2016).

Withania somnifera's naturopathic care on anxiety symptoms when compared to other usual therapies was assessed in a study where the participants were randomly divided into two. They received the regular therapies with one group alone receiving Withania root standard (Cooley *et al.*, 2009). In a study, the rats were sleep deprived for 24hours and then were injected with *Withania somnifera* extract. This resulted in shortened sleep latency, increased non rapid eye movement and sleep time, decreased waking and total sleep time (Kumar & Kalonia, 2008). Thus proved the effect of *Withania somnifera* root extracts possible interaction with GABAergic modulators which plays an important role in sleep wake cycle. This was clearly assessed by the antagonistic mechanism against GABAergic modulators picrotoxin and mucimol (Kumar & Kalonia, 2008). Withania ability to treat male infertility due to stress was proved in a trial comprising of 60 fertile men in control group and 60 infertile men in the treatment group (Shukla *et al.*, 2009). Withania treatment decreased stress and increased the antioxidant level and overall semen quality in individuals. Added to this, the treatment resulted in the pregnancy of the partners of 14% of the patients (Mahdi *et al.*, 2011).

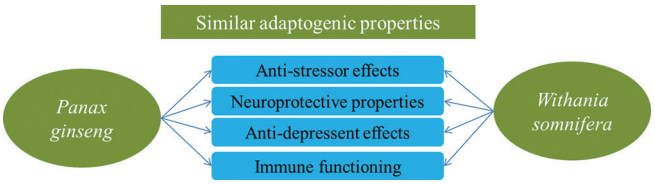

Figure 3: Similar adaptogenic properties between two ginseng plants

SIMILARITIES BETWEEN *WITHANIA SOMNIFERA* AND *PANAX GINSENG*

Withania somnifera especially the root consists of secondary metabolite compound known as withanolides which is found to have extraordinary therapeutic and medicinal properties. Withanolides are steroidal alkaloids and steroidal lactones which resembles both in their appearance and action to the active constituents of Korean ginseng (*Panax ginseng*) known as ginsenosides. Studies have shown that *Withania somnifera* provides cardio protection in ischemic rats which is similar to the adaptogens in *Panax ginseng* (Figure 3). Heart weight and glycogen in myocardium is found to significantly increase intensifying the anabolic process and enhancing the contractile duration (Mohanty *et al.*, 2004; Lim *et al.*, 2013). The extracts of both the *Withania somnifera* and *Panax ginseng* were compared for some of the therapeutic properties which also includes chronic stress (Bhattacharya & Muruganandam, 2003; Seenivasagam *et al.*, 2011). It showed that both Ashwagandha and *Panax ginseng* reversed the chronic stress and its other related effects such as Ulcer, stress induced inhibition of sexual behavior, retention of learned tasks and immune suppression. *Withania somnifera* had very similar activity to that of *Panax ginseng* except that *Withania somnifera* had some extra advantages on increasing the peritoneal macrophage activity and exclude the appearance of ginseng-abuse syndrome characterized by water retention, muscle tension, insomnia and high blood pressure (Bhattacharya *et al.*, 2000; Gajarmal *et al.*, 2001). Further, the neuroprotective effects of *Withania somnifera* and *Panax ginseng* was studied in Parkinson's induced rat models. The inhibition of oxidative stress and anti-inflammatory effects were found to be significantly decreasing the progression of PD in rats (Zhou *et al.*, 2016; Vegh *et al.*, 2021).

CONCLUSION

Plants have been used as adaptogen since ancient times. In traditional medical systems such as Ayurveda, Siddha and traditional Chinese medicines celebrated the ginseng family plants as a potent adaptogen, efficient Rasayana herb and heath tonic to rejuvenate the entire heath. Further, the adaptogens are commonly used to maintain homogeneity in human/animal body via regulations of certain metabolites. Stress induced disorders like neuro-endocrine disorders, immunomodulatory diseases could be reduced using herbal adaptogens. Among them, phyto-constituents such as withanolides and ginsenosides are widely used and studied for its apoptogenic properties. These are also believed as an adjuvant that increases the quality of life of humans in post recovery of diseases, surgical and traumatic accidents.

ACKNOWLEDGMENT

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ABBREVIATIONS

NO–Nitric oxide; CNS–Central nervous system; JNK–Jun N-terminal kinase; FOXO–Forkhead box O transcription factor; HSP–Heat shock protein; ATP–Adenyl tri phosphate; ACTH–Adrenocorticotropic hormone; REM–Rabid eye movement; GABA–Gamma amino butyric acid; IFN–Interferon; IL–Interleukin; TBNK–Lymphocyte subset (T, B, NK cells); GCLC–Glutamate-cysteine ligase catalytic subunit; NRF2–Nuclear factor erythroid 2; HT–Hydroxytryptamine.

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Research Article

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Influence and distribution of lead nitrate on growth and secondary metabolite accumulation in *Withania somnifera* (L.) Dunal *in vitro* shoots

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ABSTRACT

Withania somnifera is a medicinal ayurvedic plant native to India. Lead (Pb) is a toxic heavy metal which has no biological function in plants. The aim of the present study is to investigate the influence of heavy metal Pb on *W. somnifera* at cellular level under controlled conditions. The role of Pb on shoots of *W. somnifera* was determined in terms of biomass, secondary metabolite production (High Performance Thin Layer Chromatography) and phytochemical quantification. Influence and accumulation of Pb at the cellular level were recognized using UV visible spectroscopy, field emission scanning electron microscope and x-ray diffraction analysis. The current result showed that the concentration up to 2.4 mM Pb for 7 days of exposure showed higher biomass and withaferin A yield compared to control shoots. On the other hand, other secondary metabolites such as flavonoids, phytosterols and phenols levels were reduced compared to control shoots. In the morphological study, Pb concentration in the analysed sample was determined as 2%. The probability of the nanoparticle nature of the bioaccumulated lead also verified using spectroscopy and x-ray diffraction analysis. To date, we are the first to report on the influence of Pb on *in vitro* shoot cultures of *W. somnifera*.

KEYWORDS: *In vitro* shoots, Lead nitrate, *W. somnifera*, Secondary metabolites, Field emission scanning electron microscopy

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INTRODUCTION

Heavy metals are generally present in the soil, plant bodies and aquatic ecosystems at a higher level, however, smaller proportion are also found in the atmosphere. Many heavy metals (Nickel, Iron, Copper and Manganese) are considered as essential metals for plant growth, but the toxicity of these metals in plants is species dependent (Jan & Parray, 2016). Certain non-essential heavy metals have been used as abiotic elicitors to elicit the accumulation of specialized metabolites in plants. It has been reported that plants which undergo heavy metal stress produce higher secondary metabolite content which is positively correlated to heavy metal concentration but only to a certain point (optimum concentration), beyond which the metabolite production decreases (Rout *et al.*, 2019). Generally, Pb causes a significant reduction in seed germination rate, thickens cell walls, growth inhibition and chlorosis in plants (Yolcu *et al.*, 2021; Collin *et al.*, 2022) Certain plants have hyper accumulation capability to withstand and grow in metal rich soils *viz.* mining area or at industrial area (Castañares & Lojka, 2020). These hyper accumulators have evolved a few specialized capabilities to withstand heavy metal stress and toxicity such as bioaccumulation of metals in their greener regions, sequestering metals to vacuoles/vesicles of the cells, binding to phytochelatins or metallo enzymes and activation of numerous antioxidants (Hakeem, 2015; Collin *et al.*, 2022).

W. somnifera is a well-known Indian medicinal and Ayurvedic plant with adaptogenic properties and hyperaccumulating capability (Maharia *et al.*, 2010). *W. somnifera* has a higher metal bioaccumulation ability towards Lead (Pb), Chromium (Cr) and Cadmium (Cd). Among analysed metals, Pb was the highly accumulated metal and it was found mainly in the aerial parts (stem & leaves) of *W. somnifera* (Khan *et al.*, 2007; Balafrej *et al.*, 2020). The report shows that Pb mainly precipitates in the cell wall of the root and only free Pb ions can be transported to other parts of the plant via xylem and phloem cells (Mathur & Chauhan, 2020). Therefore, *in vitro* liquid medium and shoot

cultures (Parameswari *et al.*, 2017) were used for the Pb exposure study which facilitates free availability of Pb to the shoots. Hence the present study was focused to study the uptake of lead nitrate ($Pb(NO_3)_2$) by *in vitro* shoot cultures of *W. somnifera* and its influence on withaferin A (an important secondary metabolite native to *W. somnifera*) and the accumulation of lead in leaves.

MATERIAL AND METHODS

Plant Material

Seeds of *Withania somnifera* (L.) Dunal Var. JA20 (Jawahar-20) were collected from the University of Agricultural Sciences, Bangalore. Surface sterilized seeds were germinated under *in vitro* conditions and seedlings were maintained on half strength MS (Murashige & Skoog) medium containing 2% sucrose and 0.8% agar. The shoot obtained from the germinated seedlings was used as the source of explants and has been inoculated onto the full-strength MS liquid basal medium, cultured at 25±2 °C and a photoperiod of 16 hours for 30 days. For shoot multiplication, 30 days old shoots were inoculated onto MS medium supplemented with 4.44 µM BAP (6-benzylaminopurine) hormone (Vinod *et al.*, 2022).

Treatment with Lead Salts

The nodal sections of *W. somnifera* were excised, trimmed at both ends and inoculated in culture bottles containing MS supplemented with BAP medium. For Pb treatment, one month old *in vitro* shoots of *W. somnifera* from MS supplemented with BAP medium were transferred to MS media containing different concentrations (T1-T5) of $Pb(NO_3)_2$ salts (Table 1). Media without $Pb(NO_3)_2$ served as a control (T0). Each experiment had 3 replicates with three explants in each. The shoots were harvested after 7 and 14 days of exposure period, weighed, shade dried at room temperature and extracted using methanol (Vinod *et al.*, 2022). The fresh shoot tissues were weighed and the growth index was calculated using the formula:

$$GI = Fresh\,weight\,of \quad \{\frac{harvested\,biomass - inoculum}{inoculum}\}$$

Quantification of Secondary Metabolites

Withaferin A stock solution was prepared in the concentration of 1 mg/mL using HPLC grade methanol. From the stock solution, the working solutions were diluted to a 1:1 ratio and stored at -4 °C until further use. High Performance Thin Layer Chromatography (HPTLC) was performed on precoated silica gel aluminium plate 60F254 (MERCK, Germany) using CAMAG HPTLC for withaferin A quantification. For HPTLC analysis, the procedure reported by Vinod *et al.* (2022) was followed. The concentrated methanol extract was further used for the phytochemical analysis. Phytochemicals such as flavonoids, phenols and steroids were quantified using the procedure reported by (Kumar *et al.*, 2014; Clemensen *et al.*, 2022).

Table 1: Concentration and treatment period for Pb exposure to *in vitro* shoots of *W. somnifera*

Treatments	$Pb(NO_3)_2$ concentration	
	7 days	14 days
T0	0 mM (control)	
T1	0.6 mM	
T2	1.2 mM	
T3	1.8 mM	
T4	2.4 mM	
T5	3.0 mM	

UV-visible Spectroscopy

The methanol extracts were further subjected to spectral scan in a UV-Visible Spectroscopy from the range of 200 to 1000 nm (UV-vis 1800 Shimadzu). HPLC methanol was used as a reference control.

X-Ray Diffraction

The X-ray Diffraction (XRD) patterns of the samples were recorded (PANalyticalX'Pert PRO XRD) using Cu Kα radiation (λ = 0.15406 Å). Line broadening analyses using the Debey-Schherer formula after accounting for instrumental Broadening revealed the presence of crystalline size in the samples.

$$D\ XRD = 0.90\lambda / \beta\ \cos\theta$$

Where, D- crystal size; λ- wavelength of the X-rays; β- full width at half maximum of the diffraction peak; θ- diffraction angle (Sahayaraj *et al.*, 2012).

Electron Microscopy Analysis

Leaves from Pb treated shoots were excised, cut into small portions (3 × 3 mm) and fixed for 2 hours at 4 °C in 0.1% (weight/volume) buffered sodium phosphate and 3% (weight/volume) glutaraldehyde at pH 7.2. The leaves were stored in buffer solution until FESEM EDAX analysis.

Statistical Analysis

The data on the effect of Pb on shoot biomass in control and treated shoots were analysed using SPSS and expressed as mean ± standard deviation.

RESULTS

Morphological Changes and Biomass Production

W. somnifera shoot cultures were treated with lead nitrate at different concentrations for 7 days and 14 days period. T1 shoots showed a slightly low growth index compared to control shoots (T0). However, among all the treated cultures, a gradual increase in growth index was observed up to T4 of 7 days and T3 of 14 days exposed shoots. In T5 shoots of 7 days exposure and T4 & T5 shoots of 14 days of exposure results in a decreased growth index compared to T0. An increase in culture period to 14 days

further reduced the growth rate at earlier stages (T1); however, a maximum growth of 1.32 was observed in T3 (2.4 mM) of 14 days (Figure 1) and further increase in Pb concentration significantly decreased the growth index (T4 & T5).

Phytochemical Analysis

The concentration of flavonoids was estimated using the method described by Clemensen *et al.* (2022). The flavonoid content of shoots grown on media supplemented with varying concentrations of lead nitrate is presented in Figure 2. Compared to control shoots (T0), the flavonoid content in Pb treated shoots was (T1-T5) found to be significantly reduced at both periods. However, among Pb treated shoots, a gradual increase in flavonoid levels was observed (T1- T4) which decreased with an increase in the concentration of Pb (T5).

The phytosterol content estimated in Pb-treated and control shoots is presented in Figure 2. Among control shoots, the concentration of phytosterols in 14 days old *in vitro* shoots of *W. somnifera* recorded significantly higher levels than in 7 days treated shoots. Among treatment groups, a significant decrease in levels of phytosterol was observed with an increase in concentration of $Pb(NO_3)_2$ when compared to control (T0).

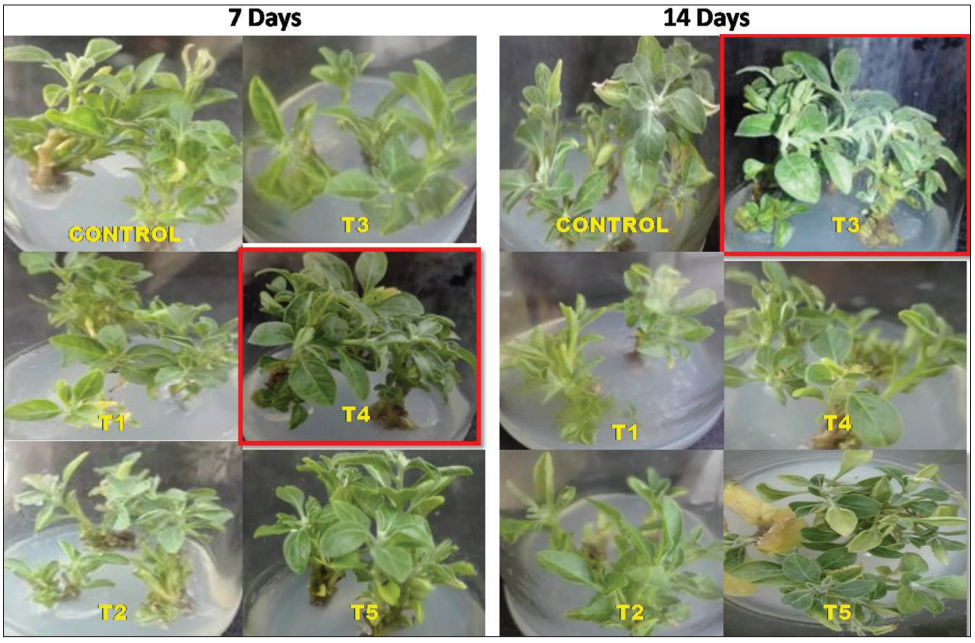

Figure 1: *In vitro* shoot cultures of *W. somnifera* exposed to different concentration of $Pb(NO_3)_2$ for 7 & 14 days

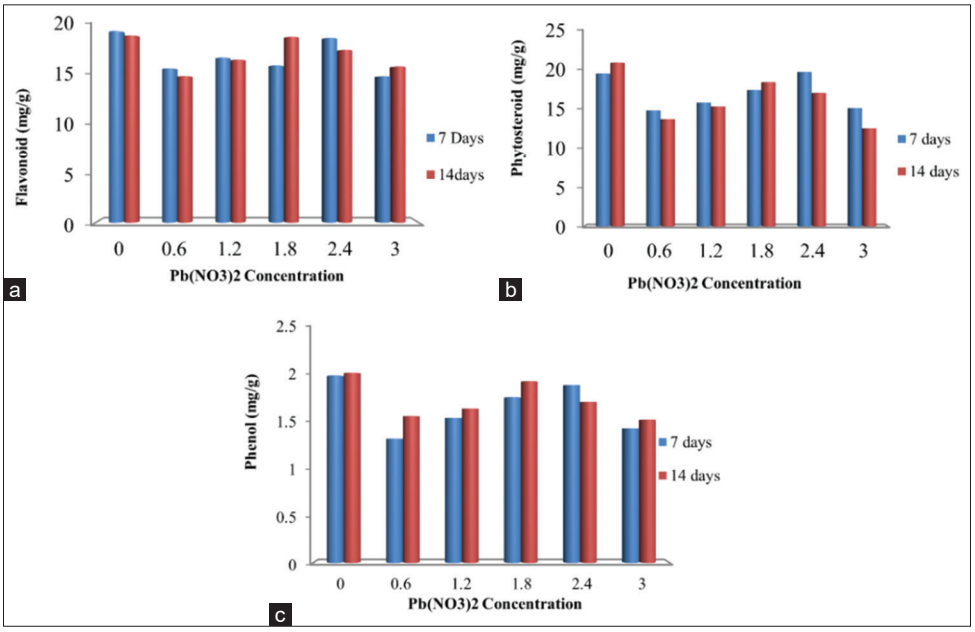

Figure 2: The figure shows the concentration of a) flavonoid, b) phytosteroid and c) phenol concentration in Pb treated shoots of *W. somnifera*

Among treated groups, a gradual increase in phytosterol content was observed in T1-T4 (14.84- 19.75 mg/g) in 7 days and T1-T3 for 14 days (13.72-18.45 mg/g) after which the levels were decreased.

The total phenol content (TPC) estimated in Pb treated and untreated (control) shoots is presented in Figure 2. Among treated groups, a gradual increase in phenol content was observed in T1-T3 (1.56-1.93 mg/g) after which the levels decreased with the maximum content of 1.93mg/g (T4) in shoots grown for 14 days. Compared with control the levels of phenol decreased significantly, but among Pb treated shoots, there was a gradual increase from 1.32 to 1.89 mg/g (T1-T4) which decreased to 1.43 mg/g (T5) for 7 days of exposure. A similar trend was observed for 14 days of exposure, with the increase in TPC levels up to T3 (1.93 mg/g) after which the TPC levels decreased significantly.

Quantification of Withaferin A

Withaferin A concentration was positively correlated to the Pb concentration up to T4 of 7 days exposure. On 14 days exposure to Pb, T3 had the higher withaferin A content (1.37 mg/g) after which (T4 & T5) a reduction in withaferin A content was observed (Figures 3 & 4). The accumulation of withaferin A was found to be 6.33-fold higher in T4 for 7 days and 4.87-fold higher in T3 for 14 days exposure shoots compared to control *in vitro* shoots.

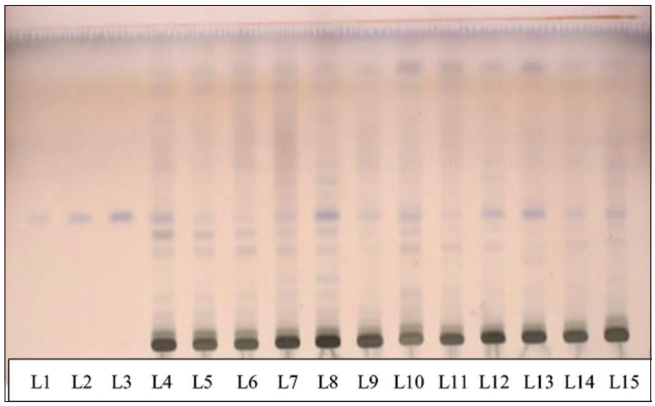

Figure 3: HPTLC separation of *W. somnifera* Pb treated and control shoot samples. Lane 1-3 = Withaferin A (standard); Lane 1-6 = Pb7 days treated shoot samples (T1-T5); Lane 6-12 = Pb14 days treated shoot samples (T1-T5)

UV-visible Spectroscopy

The results showed significant changes in the metabolite peaks of test samples compared to control. Both control and test samples exhibited similar distributions of peaks at 290 nm, 295 nm, 410 nm, 465 nm, and 665 nm. However, the intensity of these peaks was differed among test samples. Compared to control, Pb treated test samples especially at high concentration (T3-T5) had higher peak intensity at both treatment period (7 & 14 days).

Field Emission Scanning Electron Microscopy (FESEM)

FESEM micrograph (Figure 5) shows the presence of several elements which are essential for the growth of the shoots cultured *in vitro* among which 2% Pb was identified in the leaves of analysed sample which was further confirmed by the EDAX spectrum. Elemental mapping also showed the presence of carbon 41%, oxygen 33%, magnesium 2%, potassium 12%, chlorine 4%, phosphorus 3% and cobalt 1% along with Pb. Further, there were no nanostructures or any distinct surface morphology was observed in FESEM micrographs.

X – Ray Diffraction Analysis

The XRD image of lead nitrate treated *in vitro* leaves of *W. somnifera* (T5) is shown in Figure 6. The major peak at 2θ value of 27.35 was confirmed as Pb. The crystalline size of the powder is calculated to be about 8.778 nm using the formula.

DISCUSSION

Approximately 2 g of one month old shoots grown in MS supplemented with BAP medium were transferred to MS basal medium. This was done to ensure that the explants used for the present study do not have any residual influence of hormones and whatever response we observe will be solely due to the treatment of $Pb(NO_3)_2$. The biomass accumulation patterns in Pb exposed shoots (T2-T4) were significantly increased compared to control (T0).On treatment with Pb salts, the *W. somnifera* shoots exhibited increased metal tolerance and resistance which resulted in the increased growth index at both 7 days and 14 days treatment periods. However, our observation is contrary to many studies which proved that Pb was negatively correlated with plant growth after a certain concentration (Collin *et al.*, 2022). Although lead (Pb) is a toxic

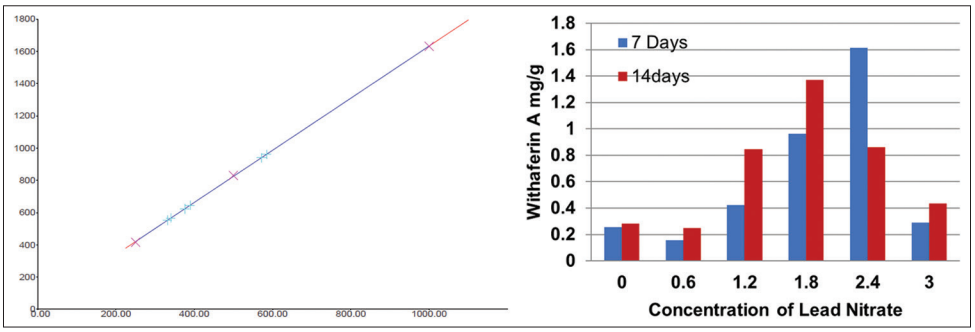

Figure 4: Withaferin A accumulation in Pb exposed and control shoots of *W. somnifera* for 7 and 14 days

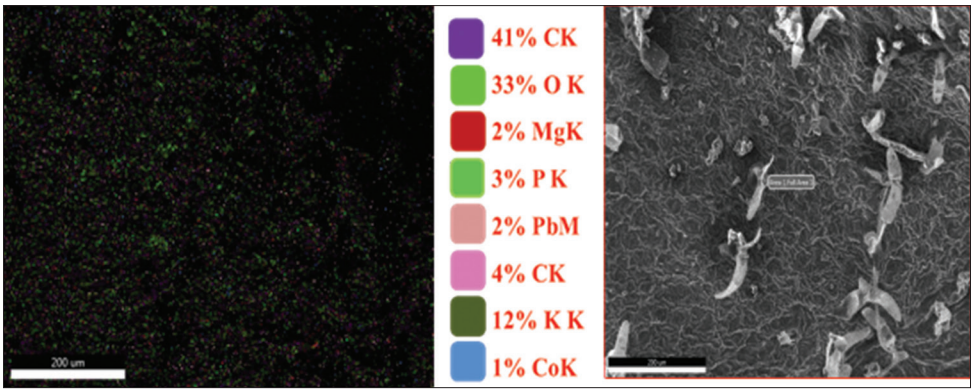

Figure 5: Elemental mapping and Pb accumulation pattern in T5 shoot of *W. somnifera*

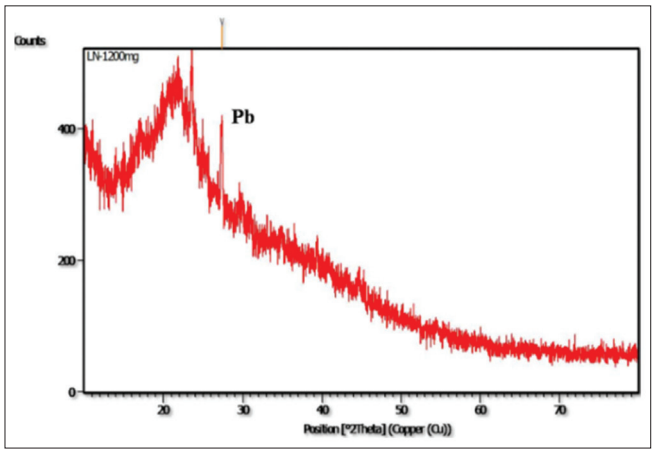

Figure 6: XRD spectrum of T5 shoots for 14 days exposure

heavy metal, *W. somnifera* showed significant growth in media containing Pb up to a concentration of 2.4 mM (T4) for 7 days and 1.2 mM (T2) for 14 days of exposure (Table 2). A similar response was observed in *in vitro* cultures of *Datura inoxia* which showed endurance up to 45 mg/L lead concentrations with a slight decrease in the number of shoots with increasing concentrations (Waoo *et al.*, 2014). The ability of these plants to tolerate stress might be due to their potential to selectively absorb the necessary nutrients and sequester non-essential metals to vacuoles or other organelles for maintaining the system homeostasis (Peng & Gong, 2014).

Following the isolation of the active ingredients, they can be incorporated into the modern medicine system to develop effective formulations for therapeutic purposes. Several specific reactions operating communally are responsible for the production of secondary metabolites in plants (Mansoori *et al.*, 2020). The medicinal value of these secondary metabolites is due to the presence of reactive components that produce a definite biochemical reaction in humans (Natarajan *et al.*, 2022). Flavonoids comprise the most common group of plant polyphenols and provide much of the flavour and colour to fruits, vegetables and have potent antioxidant and free-radical scavenging activities.

During Pb treatment, Pb increases the flavonoid content at earlier concentrations but decreased flavonoid content was

Table 2: Growth index of Pb exposed shoots of *W. somnifera*

Treatment	7 days	14 days
T0	1.25±0.011	1.44±0.015
T1	1.10±0.015	1.04±0.002
T2	1.67±0.030	1.31±0.005
T3	1.89±0.039	1.32±0.010
T4	1.93±0.031	1.15±0.019
T5	1.03±0.012	1.09±0.011

observed at higher Pb concentration (T5). Similar results were observed in the flavonoid content of shoots subjected to 14 days treatment. Ibrahim *et al.* (2017) has reported a decrease in the phenol and flavonoid content with an increase in the concentration of cadmium and copper. A similar result was also observed in the phytosterol content of Pb treated shoots in the present study. The toxicity of Pb affects the phytosterol level at higher concentrations and prolonged periods. TPC level was also increased during Pb treatment and increased TPC content was observed at T4 of 7 days exposure and T3 of 14 days exposure. TPC analysis on leafy vegetables and fruits was done by Chandra *et al.* (2014). They reported that basil (32.50 mg/g) and chard (41.15 mg/g) contain higher TPC than other leafy vegetables. In a study reported by Saini and Gupta (2017), the seedlings of mung beans were treated with Zn, there was an initial decrease till 100 ppm followed by a step increase at 300 ppm (1.793 mg/g) and followed by a further decreased (2.215 mg/g) at 1000 ppm of TPC were observed, when compared with untreated seedlings.

HPTLC analysis was performed in the current study to quantify withaferin A content in the methanol extract of $Pb(NO_3)_2$ treated *in vitro* shoots of *W. somnifera*. One of the important secondary metabolites in *W. somnifera* is withaferin A has higher pharmaceutical value than other withanolides (Natarajan *et al.*, 2022). Pb treatment was positively correlated with withaferin A content till a specific concentration. Increased concentration of withaferin A was observed at T4 of 7 days and T3 of 14 days with T4 of 7 days exposure being higher compared to control (T0) and other treatments. The present result was in correlation with Sivanandhan *et al.* (2012), in their study approximately 7-fold increase in the withaferin A contents was observed when *W. somnifera* cultures treated with aluminium chloride as an elicitor. Salicylic acid was used as an elicitor for *W. somnifera in vitro* callus cultures where 20-fold increases in withaferin A

was reported. Thus, the results show the heavy metal stress on the *in vitro* cultures of *W. somnifera* increases the secondary metabolite content, especially withaferin A.

UV visible spectrophotometer was used to identify if the Pb was absorbed and accumulated as ions or particulates in shoots of *W. somnifera*. The standard Pb ion in the solution showed a peak at 295 nm. The samples of Pb treated shoots also showed peaks at the same wavelength; however, it might be due to the presence of high protein content (absorbance at 290 nm) in the Pb treated shoots or it might be from a protein molecule that is present on the surface of the reduced Pb element. The probability of the presence of nanoparticles in the Pb treated samples is also investigated in further studies. It has been reported that the Pb nanoparticles were synthesized through green synthesis using *Zingiber officinale* extract and gave peaks around 239 nm and 335 nm during UV visible spectroscopy analysis (Delma & Rajan, 2016). Another study on *Cocos lucifera* extract and Pb nanoparticles synthesis followed by spectroscopic analysis showed peaks at 212 nm (Elango & Roopan, 2015). Thus, the presence of peaks varied from 200-350 nm for green synthesized Pb nanoparticles which is due to their size and shape. Therefore, it might be possible that the accumulated Pb may reduce to nanoparticles in live plants of *W. somnifera* which could give its UV absorption at 295 nm (Figure 7). The peaks found at 465 nm and 665 nm were found to be chlorophyll a and b, peak identified at 410 nm shows the presence of β-carotene. Chlorophyll a & b and β-carotene levels were increased in Pb treated shoots for 14 days of exposure than in control shoots (Figure 7) which may be due to the Pb stress on the tissues of *W. somnifera*. However, on 7 days of Pb exposed shoots, there was no significant difference was observed in the control shoots except T4 shoots had stronger chlorophyll a & b and β carotene peaks (Figure 7).

Electron microscopy images were measured and topographical analysis was performed based on the surface analysis of leaf tissues of a T5 shoots of 14 days exposure. The analysed samples were found to contain 2% Pb along with other elements like O, Mg, K, Cl, P, and Co. The determined 2% Pb was found to be scattered all over the sample which further confirms that the Pb was absorbed, transported and stored in the leaves of *W. somnifera* (Figure 5). While, the nature of the accumulated Pb in the leaf could not be studied using FESEM. However, the Pb peak at 2.4 kev in the EDAX spectrum shows the probability of nanoparticle formation in the live plant samples (Figure 8). A similar result was observed in (Miri *et al.*, 2018; Diba *et al.*, 2021). In conclusion, the presence of Pb in leaf tissues of *W. somnifera* might be from diffusion, transportation and accumulation of Pb from the media to the leaves.

The report on *Sesbania drummondii* confirms that the absorbed Pb cannot be leeched out from the plant even if they put in the control condition (Hu *et al.*, 2015). Alfaraas *et al.* (2016)

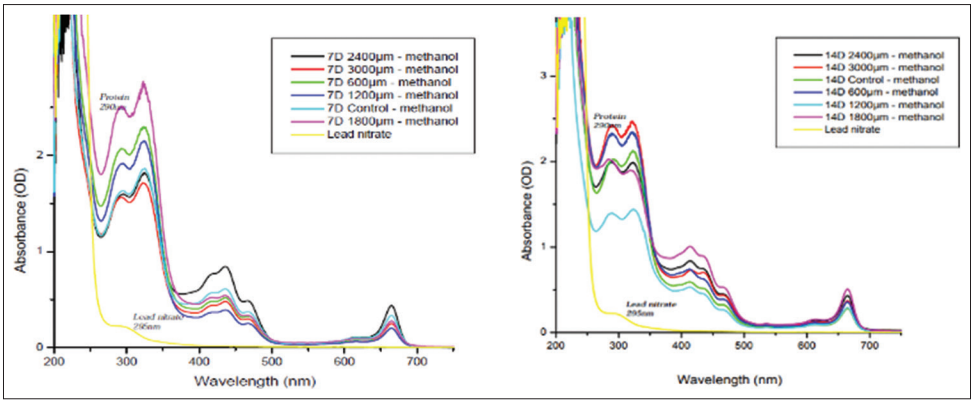

Figure 7: UV-Visible Spectrum of Pb treated shoots for 7 days (left) and 14 days (right) exposure

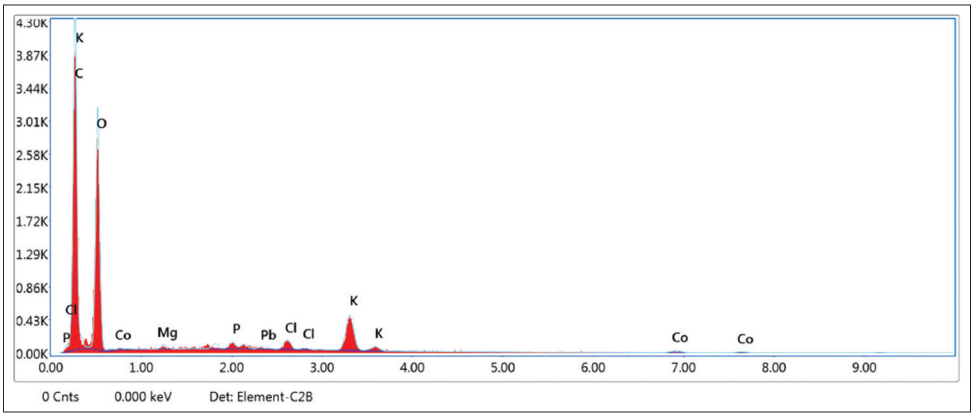

Figure 8: EDAX spectrum of T5 shoot

reported that the effect of heavy metals such as Pb and Cd on the leaf and root of paddy plants via cross sections. They reported that accumulation of Pb and Cd in paddy plants was in a concentration dependant manner and also at higher concentrations, metal causes physiological changes like chlorosis of leaf and reduction in growth. Further, Pb adsorption, translocation and hyper accumulation were detailed in crop species by Wang *et al.* (2021).

Crystalline size and structural properties of accumulated Pb were revealed using X-ray diffractions. XRD studies were carried out with Cu – κα radiation (κ – 0.154 nm) and 2 theta ranged from 20° to 80°. From Figure 6 Pb peak was observed 2θ values of 27.3 and using 2θ value crystalline size was calculated to be 8.78nm. A similar result was reported by (Delma & Rajan, 2016), and reported that Pb nanoparticles synthesized using *Zingiber officinale* extract had diffraction peaks at 2θ values of 38.140, 19.720 and 32.380 and the average particle size of Pb nanoparticles was found as 3nm according to Debye Scherrer equation. In addition, 2θ values for Pb nanoparticles synthesized by bacterial strains of *Bacillus toyonensis* were 26, 30, 43 and 51 (Mathew & Krishnamurthy, 2018). Similar result was identified from our study that the Pb from *in vitro* shoot of *W. somnifera* showed 2θ values at 27.35.

CONCLUSION

In the present study, the maximum biomass and withaferin A content was observed in 7 days $Pb(NO_3)_2$ exposure when compared to control and 14 days treated shoots of *W. somnifera*. High withferin A content (1.615 mg/g) was quantified in T4 (2.4 mM) shoots of 7 days exposure and showed 6.33 fold increase of withaferin A. UV visible spectroscopy confirmed the presence of Pb peak at 295 nm when compared with standard $Pb(NO_3)_2$ solution. Electron micrograph confirms the absorption, transportation and accumulation of Pb (2%) in the leaf tissues of Pb treated shoots (T5 at 14 days). However, the elemental nature of the Pb in the sample was not identified in either instrumentation. However, according to EDAX and XRD results, there is a high probability for the presence of Pb nanoparticles in the live leaf tissues. To conclude, *W. somnifera* can survive heavy metal stress. Though toxicity affects its growth on prolonged exposure it also acts as a good elicitor for withaferin A production. Further, ultra-structural analysis on leaf/stem tissues are needed to analyse the presence of nanoparticles in live tissues.

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An investigation on reduction capability of lead and its influence on withanolides in *in vitro* shoots of *Withania somnifera* (L.) Dunal

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Withania somnifera

ABSTRACT

Green synthesis of nanoparticles using biological extracts/live organisms has been the best substitute for chemical/physical synthesizing methods. Live plants can convert simple metal ions into metal nanoparticles using their intrinsic metabolic processes. In addition, medicinal plants with a hyperaccumulation potential also have high reduction potential which can be utilized for *in planta* synthesis of metal nanoparticles. Lead (Pb), a non-essential heavy metal, is known to stimulate various biological processes in plants at higher concentrations. Therefore, a medicinal plant with a hyperaccumulation potential was selected to investigate its potential for *in planta* reduction of Pb. In the present study, *in vitro* grown 45 days old *Withania somnifera* (L.) Dunal shoots were exposed to lead acetate ($PbAC_2$) at varying concentrations (acute: 300–4800 μM; chronic: 50–800 μM) for 12–48 h and 4–12 days, respectively. It revealed that cultures grown in 800 μM $PbAC_2$ for 12 days of exposure (C5) recorded significantly higher biomass (17.28 g) and increase in the divalent metal ions such as calcium from 1412 (control) to 1787 mg kg-1 (C5), potassium 21,109 (control) to 24,779 mg kg-1 (C5), and iron from 11 (control) to 82 mg kg-1 (C5), with lead (Pb) accumulation reaching up to 405 mg kg-1 (C5). Compared to the control shoots (0.783, 0.805 & 1.3 mg g-1), C5 shoots positively correlated with secondary metabolites namely withaferin A (3.14 mg g-1) and withanolide A (0.960 mg g-1) production and negatively with withanone (1.027 mg g-1). Also, C5 shoots had spherical shaped nanoparticles with a mean particle size of 25±10 nm which was then identified as lead nanoparticles (PbNPs). To our knowledge, the present study is the first of its kind where the *in planta* synthesis of PbNPs/PbONPs by *in vitro* shoot cultures of *W. somnifera* is reported along with accumulation of higher biomass and selective withanolides under Pb stress.

1. Introduction

Bio-Metallic Nanoparticles (Bio-MNPs) referred as green synthesized-MNPs are receiving considerable interest in the scientific community as it exhibits very low toxicity and higher stability in a biological system (Singh et al., 2018); on the other hand, MNPs are reported to be toxic and dangerous for human health and the environment (Baig et al., 2021). Thus, the production of Bio-MNPs from biological materials, including plant extract, algae, or bacteria, without requiring harmful agents or producing harmful by-products are advantageous (Singh et al., 2018; Shanmugam et al., 2022). These Bio-MNPs have an array of biomedical applications, including antibacterial (Bhuyar et al., 2020; Amer and Awwad, 2021), anticancer (Shanmugam et al., 2022), antifungal (Karmous et al., 2022), antidiabetic, anti-inflammatory activities (Govindappa et al., 2018) and also used as a nano-fertilizers and nano-pesticides/ herbicides (Castillo-Henriquez et al., 2020). Plants make it possible to produce nanoparticles in an eco-friendly manner and transform difficult-to-synthesize metals into nanomaterial (Parker et al., 2014). To our knowledge, until date, no studies conclusively demonstrate the synthesis of Bio-MNPs for many elements commonly studied for phytoremediation and phytomining, including lead, nickel, cadmium, chromium, and zinc. Uptake of heavy metals in hyperaccumulator plant is well studied in several investigations, however,

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their potential in synthesizing bio-MNPs and their nature of accumulation needs extensive research.

In planta formation of metal nanoparticles by live plant seedlings were reported in *Medicago sativa* (Gardea-Torresdey et al., 2002, 2003; Marchiol et al., 2014), *Brassica juncea* (Ag & Au NPs) (Harris and Bali, 2008; Marshall et al., 2007; Haverkamp and Marshall, 2009; Marchiol et al., 2014), *Festuca rubra* (AgNPs) (Marchiol et al., 2014), *Arabidopsis thaliana* (Palladium NPs) (Parker et al., 2014), *Cucumis sativus, Organum vulgare, Trifolium pratense, Lolium multiflorum* and *Helianthus annuus* (AuNPs) (Starnes et al., 2010). According to reports, the chloroplast is the site for metal reduction in living plants with reducing sugars in the chloroplast acting as the reagents (Beattie and Haverkamp, 2011). The presence of secondary metabolites determines the reduction potential of host plant influencing the synthesis of nanoparticles. Samsoon et al. (2022) reported plant extracts or live plants can synthesize MNPs, acting as capping and reducing agents (Samsoon et al., 2022). The ability of a plant to acquire metals from soil or medium is correlated with higher levels of secondary metabolites (Nasim and Dhir, 2010; Anjitha et al., 2021), which promotes their survival by enabling high tolerance limits for metals (Jampasri et al., 2021). Therefore, a medicinal plant, *Withania somnifera* (L.) Dunal (*W. somnifera*) with hyperaccumulating capability and a few important secondary metabolites (Withanolides), was selected for this study. Further, *in vitro* cultures were chosen to study the plants' bioaccumulation capability in a controlled environment. Field-grown plants may suffer from environmental factors interfering with the selected metal bioaccumulation. Therefore, *in vitro* shoot cultures of *W. somnifera* were selected for the current study.

Several studies are reported on the elicitation of *in vitro* cultures of *W. somnifera* employing metals including cadmium (Mishra et al., 2014), iron (Rout et al., 2015), copper (Rout et al., 2013), aluminium (Sivanandhan et al., 2012), arsenic (Siddiqui et al., 2015) and zinc (Rout et al., 2019) and its influence on secondary metabolism. However, the nature and fate of those elicitated metals within plant cells/tissues have not been studied yet. Further, the age of the plant also positively correlated with the concentration of metal accumulation in the plants (Rodriguez et al., 2007). Thus, the study was constructed to determine the metal accumulation potential of 45 days old *in vitro* shoot cultures of *W. somnifera* and to identify its reduction capability. Pb is a harmful heavy metal that leaches into the environment through industrial waste, battery recycling enterprises, mining, etc. Despite the fact that Pb has four electrons in its valence shell, the most common oxidation state is +2 rather than +4 since only two of the four electrons actively ionize (Blanco et al., 2021). Pb may be quickly transferred via (Two valances) ion transporters due to its ionization properties. The range of Pb levels in polluted soil is 50 to 1800 ppm. The average Pb level in Indian soil is about 200 ppm (Parth et al., 2011; Patel et al., 2022). Hyperaccumulator plants growing in contaminated soils may accumulate Pb in the form of metallic nanoparticles. In the light of above facts, the objectives of this study were to evaluate Pb tolerance in *in vitro* shoot cultures of *W. somnifera*, its reduction in to PbNPs/PbONPs and its influence on growth and secondary metabolite accumulation.

2. Materials and methods

2.1. Plant materials

The seeds of *W. somnifera* "Jawahar 20" were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Shoots (length ~5–7 cm) containing 2–3 internodes from 4 weeks old *in vitro* cultures was used for the study as described by Vinod et al. (2022).

2.2. Establishment of shoot suspension culture

For biomass accumulation and withanolides production, 25–30 days old, shoot tips/apical buds of the explants from semi-solid BAP media were sliced off. The remaining explants were transferred to a 30 mL liquid MS medium containing BAP. The suspension cultures were maintained at 50 rpm on a rotary shaker lit by fluorescent lamps, providing an average photo-synthetically active radiation (PAR) at the top of the shoot cultures of 25 μmol m^{-2} s^{-1} with a 16 h photoperiod and temperature maintained at 25±2 °C. Shoots were maintained in a BAP suspension medium for two weeks before metal treatment.

2.3. Treatment with $PbAC_2$

Forty-five days old *in vitro* shoots were weighed before the treatment and inoculated in MS medium supplemented with 1 mg/L BAP (6-benzylaminopurine) and varying concentrations of $PbAC_2$ for acute and chronic treatment and maintained for a specified time period. For acute toxicity studies, shoots were exposed to $PbAC_2$ concentration of 300, 600, 1200, 2400 and 4800 μM for shorter treatment period of 12, 24, and 48 h, whereas for chronic toxicity studies, $PbAC_2$ concentration of 50, 100, 200, 400, and 800 μM for a longer treatment period of 4, 8, and 12 days were chosen. The concentrations for acute and chronic toxicity studies were determined based on the optimum concentration of Pb in Indian contaminated and non-contaminated soils, respectively (Parth et al., 2011). $PbAC_2$ (≥99 %) stock solutions were prepared by dissolving the $PbAC_2$ in an autoclaved double distilled water and used appropriately to MS media supplemented with 1 mg/L BAP. Following the exposure period, shoots were harvested and weighed, and biomass was calculated.

2.4. Elemental analysis

The dried *W. somnifera* tissues (control *in vitro* shoot, Pb treated shoot (C5), field grown shoot) were used for Inductively Coupled Plasma Mass Spectrometry (ICPMS) (Thermo Fisher iCAP RQ ICPMS) elemental analysis. Eight essential metals, four essential heavy metals, and ten non-essential heavy metals were analysed by following standard protocol of the manufacturer. The powdered sample was digested using the wet digestion method in Anton Paar Mutiwave GO microwave digester. Precisely 0.2 g sample was ground into powder, then soaked in a digestion vessel with 6 mL of HNO_3 (≥69 %) (67–70 % for trace metal analysis) for 30 min. The samples were digested at 453 K and then made up to 50 mL in a volumetric flask. The blank solution was prepared the same way, except that no sample was added. The samples were mixed thoroughly using a vortex shaker and analysed using ICPMS for minor and trace elements. The metal concentration detected in the plant samples were compared with legal reference values for normal and toxic concentrations of heavy metals in plants are presented in Table 1.

2.5. Plant tissue processing and electron microscopy analysis

Ultra-structural analysis was performed by bright field transmission electron microscopy (TEM) (FEI, Tecnai Sprit G2, Netherlands). For TEM analysis, the protocol reported by Jain et al. (2014) was followed with slight modifications. Briefly, 50 mg fresh young leaves (2nd leaf from shoot apical meristem) were cryo-grounded and suspended with 2 mL HPLC-grade water. A 0.5 mL aliquot of the shoot extract was pulse-sonicated on ice (10 s at 95 % amplitude followed by 15 s rest with 5 pulses per cycle) with a 3 mm standard probe of the VCX500 Ultrasonic Processor with anti-noise cabinet (Sonics & Materials). The probe temperature was maintained at 20 °C. After each sonication cycle, the shoot extract was centrifuged at 4 °C for 30 s at 10,000 rpm in a table-top centrifuge (Rota R-V/Fm, Plasto Crafts). The sonication-centrifugation cycle was repeated three times to increase the tissue lysis. At the final cycle, the sonicated extract was pelleted by centrifugation, and 10 μL was transferred onto the

Table 1
Elemental composition and its maximum permissible limit in medicinal plants.

S. No.	Parameters	FS (ppm)	IS (ppm)	C5 (ppm)	Permissible Limit (ppm)
		Essential Metals			
1	11B (Boron)	31.44	21.26	15.001	Nil
2	24Mg (Magnesium)	7895.97	1282.317	769.07	Nil
3	31P (Phosphorus)	1941.08	3679.81	1189.09	Nil
4	39 K (Potassium)	27,396.51	21,109.4	24,779.14	Nil
5	44Ca (Calcium)	8689.18	1411.68	1786.92	Nil
6	55Mn Manganese)	41.16	195.55	125.74	Nil
7	57Fe (Iron)	10.50	11.20	82.102	Nil
8	95Mo (Molybdenum)	0.331	2.803	1.199	Nil
Essential Heavy Metals					
1	60Ni (Nickel)	0.987	0.003	0.079	1.5 (WHO,2005)
2	63Cu (Copper)	8.197	2.027	0.501	20 (WHO/FDA)
3	66 Zn (Zinc)	15.59	42.23	21.27	50 (WHO/FDA)
4	48Ti (Titanium)	3.667	Nil	Nil	Nil
Heavy Metals					
1	52Cr (Chromium)	0.2076	0.0076	0.0018	2 (EP)
2	59Co (Cobalt)	0.1635	0.1452	0.149	5 (USP)
3	75As (Arsenic)	0.0162	0.0045	0.003	10 (WHO/FDA)
4	111Cd (Cadmium)	0.0196	0.0055	0.003	0.2 (WHO/FDA)
5	202 Hg (Mercury)	0.0002	0.004	Nil	1 (WHO/FDA)
6	107Ag (Silver)	0.015	Nil	Nil	Nil
7	208 Pb (Lead)	0.1132	0.028	405.89	10 (WHO/FDA)
8	51 V (Vanadium)	0.03	Nil	Nil	30 (US EPA)
9	7 Li (Lithium)	0.026	Nil	Nil	0.075
10	121Sb (Antimony)	Nil	Nil	Nil	Nil

FS – Field shoot of *W. somnifera*
IS – *in vitro* shoot of *W. somnifera* / control
C5 - *in vitro* Pb treated shoot. EP – European Pharmacopeia
WHO/FDA - World Health Organization / Food and Drug Administration
US EPA - United States Environmental Protection Agency
USP – United States Pharmacopeia.

400-mesh copper-coated grids. Point and region analyses were performed at 120 kV using transmission electron microscope. The energy dispersive x-ray spectroscopy (EDAX) spectra were recorded, and micrographs were captured at 40 keV for 30–40 s.

2.6. HPTLC profiling of withanolides

A total of 0.5 g of dried shoot powder was taken, and 100 % methanol was used for the extraction. The extraction procedure was done per the protocol by Senthil et al. (2015). The pooled extracts were filtered and evaporated to dryness in a rotary vacuum evaporator (Roteva, Mumbai) using water bath at 48±1 °C and 200 Pascal. The remaining residue was re-dissolved in 5 mL HPLC-grade methanol (≥99.8 %) and centrifuged at 10,000 rpm for 5 min. The supernatant was filtered through a 0.45 μm membrane filter and stored at 5 °C. For High Performance Thin Layer Chromatography (HPTLC) analysis, the protocol reported by Vinod et al. (2022) was followed. For the stationary phase, silica gel-coated TLC aluminium sheets of 20 × 10 cm (60 F_{254}, Merck, Germany) were used. For the mobile phase, a freshly prepared solution containing toluene (≥99 %), ethyl acetate (≥99 %), and formic acid (≥85 %) in the ratio of 5:5:1 v/v was used. The slit dimension of 5 × 0.1 mm and a scanning rate of 20 mm/s were employed. The standard peaks of withaferin A, withanone, and withanolide A were used as reference peaks and compared to the sample peaks to quantify the withanolides at *in vitro* and field samples of *W. somnifera*.

2.7. Statistical analysis

The data on the effect of $PbAC_2$ on shoot biomass and yield of withaferin A (Supplementary file2) in control and treated shoots were analysed and expressed as mean ± standard error of the mean. Multivariate analysis (Duncan's multiple range test) at $p \leq 0.05$ was used to separate mean values using SPSS (version 16.0).

3. Results

3.1. Influence of Pb exposure on growth characters

The treatment of $PbAC_2$ to the shoot suspension cultures of *W. somnifera* resulted in faster growth of shoots compared to control. The growth characteristics of *in vitro* shoot cultures of *W. somnifera* in response to Pb was observed for both short duration at higher concentration (acute toxicity) and long duration for low concentration (chronic toxicity). Both treatments showed no visible toxicity symptoms though at higher concentration darkening of leaves were observed (Supplementary file1). A 12 h exposure period to high concentration of Pb (A1-A5) resulted in decrease in growth (Fig. 2) whereas, on extending the exposure time to 24 and 48 h showed no significant difference in growth conditions, which may be due to the onset of adaptive mechanism of shoots' to Pb treatment. Whereas, treatment of *in vitro* shoot cultures to low concentration (C1-C5) of Pb for longer period up to 12 days showed a significant increase in biomass. A highest growth of 17.28 g was observed in C5 over a 12-day period which is 2.12 times over than control. On longer exposure periods with a low concentration of Pb, leaves were healthy, pale green with fully/partially expanded lamina (Fig. 1), which remained constant throughout the exposure period.

The evaluation of *W. somnifera* shoot growth parameters suggests that Pb has no adverse effects on the cultures at concentrations up to 800 μM (C5) for 12 days. Generally, Pb exposure causes damage to the cell membrane and all the organelles to different extents depending on the plant and Pb concentrations. Even though *W. somnifera* shows hyper-accumulator capability; it has a threshold for each metal/heavy metal. We determined the maximum threshold for Pb concentration and best exposure period under *in vitro* conditions as 800 μM for 12 (C5) and hence was selected for further analysis. The pictures of acute and chronic lead acetate treated *in vitro* shoots of *W. somnifera* are given as Supplementary file1.

Fig. 1. (a) A3 shoot (1200 μM $PbAC_2$ treated for 48 h), (b) C5 shoot (800 μM $PbAC_2$ treated for 12 days), (c) Control shoot (untreated shoot of 12 days) of *W. somnifera*. Red arrow indicates the leaf darkening due to heavy metal stress.

3.2. *Elements in* W. somnifera *Pb-treated shoots*

The concentrations of Pb in the plant fractions were determined by ICP-MS analysis.

A total of 22 heavy and trace metals were analysed in Pb-treated shoots untreated dried *in vitro* shoots (control) and field shoots of *W. somnifera*, Except Sb, 21 elements were detected in all tissues. The levels of all heavy and trace metals were found to be within the

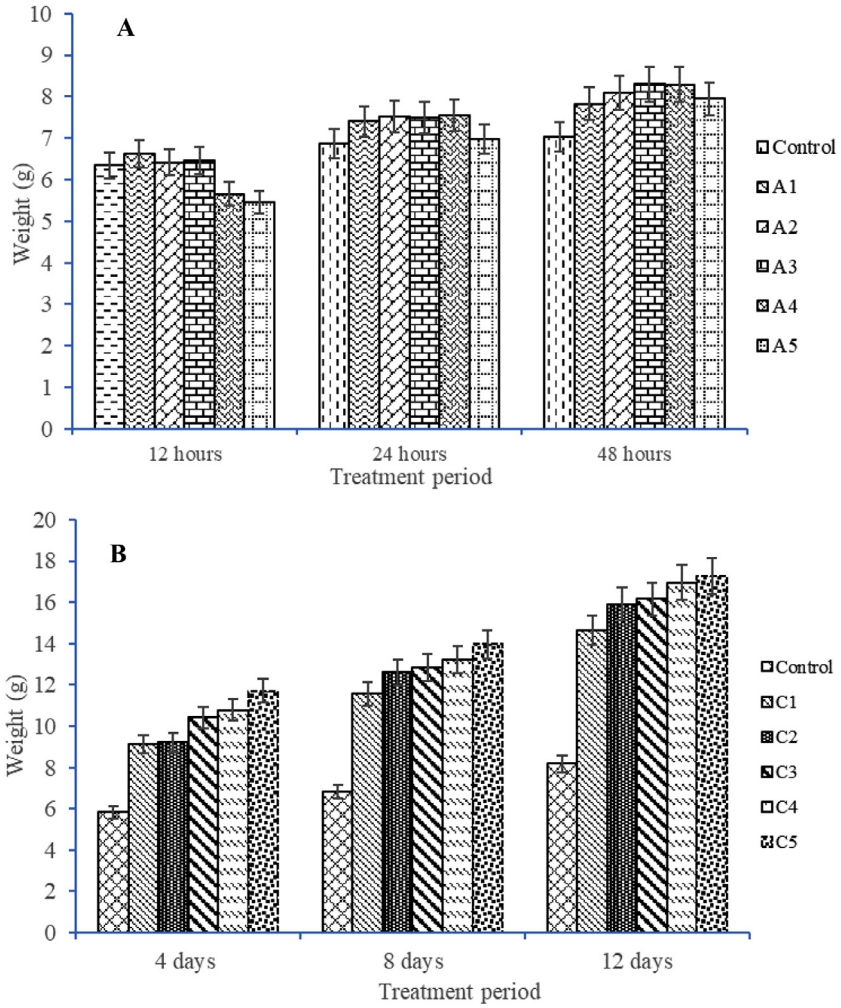

Fig. 2. Influence of $PbAC_2$ treatment on biomass of *W. somnifera*. A – Acute toxicity studies – treatment for 12, 24, & 48 h. B- Chronic toxicity studies – treatment for 4,8 & 12 days. An initial 5.0 ± 1 g shoot was inoculated on MS suspension, supplemented with 1.0 mg l^{-1} BAP & different concentrations of Pb.

permissible limit set by FAO/WHO (WHO guidelines 2007) except Pb in Pb treated C5 tissues (Table 1). An accumulation of 405 Pb ppm dry weight of tissue was recorded in C5 shoots, which is 40 times (10 ppm) above the permissible limit set by WHO (Table 1). However, an increase in certain essential metals – K, Ca, & Fe were observed in C5 shoot compared to control.

3.3. Transmission electron microscopy and EDAX analysis

Transmission electron micrograph of the mesophyll cells in a leaf of *W. somnifera* shoots treated with 800 μM for 12 days (C5) is shown in Fig. 3. Leaf sections of control and C5 shoots were analysed for the presence of nano-sized particles. C5 shoots were found with spherical and rod-shaped nano-structures in a clustered form (Fig. 3). This indicates that the nano-sized particles are present in the C5 leaf. EDAX analysis reveals a strong signal of the metallic Pb region at 2.4, 2.45 & 2.7 keV and confirms the formation of lead nanoparticles in live shoots of *W. somnifera* (Fig. 4). As can be seen in the EDAX spectra of the C5 sample in Fig. 4, most of the Pb present in the shoot samples exist as Pb^0 (metallic form) at 2.4 & 2.45kev. This confirms that the observed nano-structures are PbNPs which are reduced and accumulated by *W. somnifera* shoot tissues.

Other peaks, such as C, Ca, K, Fe, Na, O & Cl, maybe from the functional groups on the surface of the nanoparticles. These functional groups may originate from secondary metabolites (Withanolides & others), sugars, and other reducing agents from shoot cultures.

The Cu peak might be due to the Cu grid used as the sample holder. Thus, the probability of synthesized nanoparticles in the leaf tissues might be PbNPs or PbONPs (Lead oxide nanoparticles). However, it is noticeable that control shoots showed neither nano-structures (Supplementary file3) nor Pb or any other heavy metal in their system (Fig. 4). But decreased concentration of Ca, K, and Fe was observed in control shoots (Fig. 4b) compared to C5 shoots (Fig. 4a) which can be observed through elemental peak intensities (Fig. 4). This is supported by the observation in elemental analysis (Table 1).

3.4. Withanolides production in response to Pb

In the present study, Pb (heavy metal) was used as a stressor to the shoot cultures of *W. somnifera*. Heavy metal stress on plants could be counteracted through increased uptake and translocation of essential metals or by a higher production of secondary metabolites native to the selected plant species. Therefore, withanolides native to *W. somnifera* were quantified after heavy metal stress.

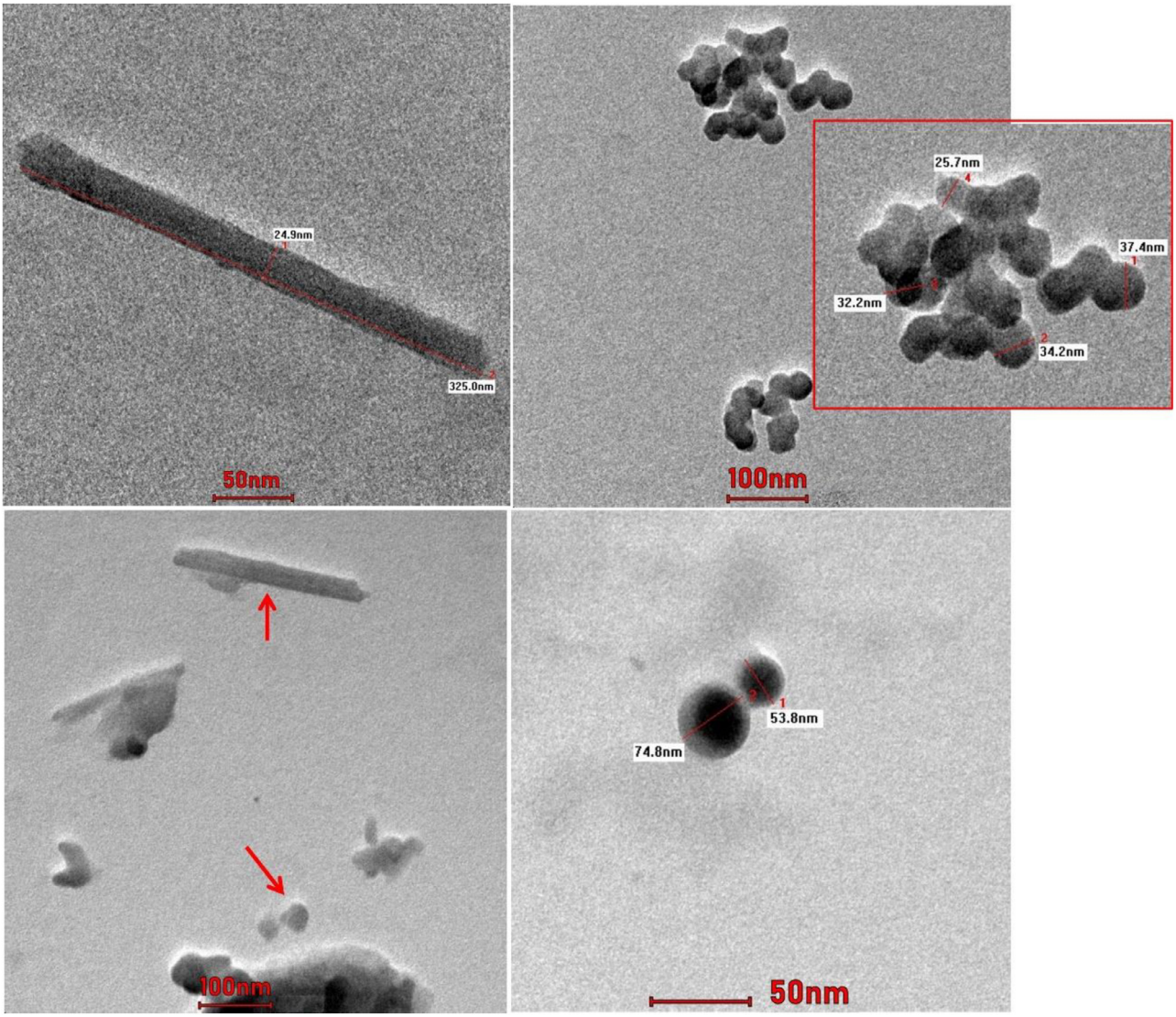

Fig. 3. The presence of PbNPs in C5 live leaf cells from *in vitro* shoot cultures of W. somnifera. Red arrow marks show that two different shaped NPs in the leaf cells, such as rod and spherical shape. Magnified picture of NPs represented within the figures.

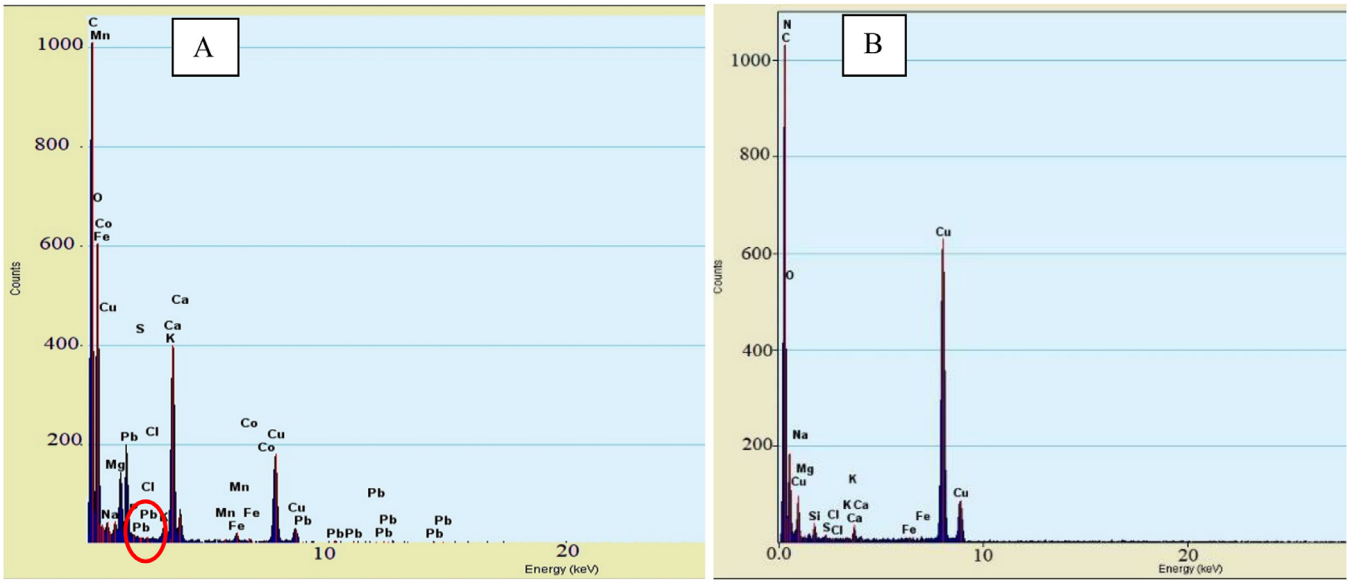

Fig. 4. EDAX spectra of A) C5 shoots and B) control shoots. The red circle indicates that the presence of Pb at ~2.4 keV in metallic form (PbNPs).

The amendment of $PbAC_2$ improved withaferin A yield in all tested concentrations (Supplementary file2). Further studies were carried out on selected Pb concentration (800 μM) treated (C5) and untreated (control) *in vitro* and field-grown shoots of *W. somnifera*. The quantification of major withanolides, namely withaferin A, withanolide A, and withanone, was carried out. Among the three tested shoots, the highest withaferin A content of 6.60 mg g^{-1} DW was detected in field-grown shoot tissues, while the lowest withaferin A 0.783 mg g^{-1} DW was detected in control *in vitro* shoots (Fig. 5 & 6).

It is evident from Fig. 6 that the withaferin A was significantly increased in Pb-treated C5 shoots (3.14 mg g^{-1}) compared to control *in vitro* shoots (0.783 mg g^{-1}) but lower than field-grown shoot samples. The C5 shoots had the highest withanolide A content (0.960 mg g^{-1} DW), followed by control i*n vitro* shoots (0.805 mg g^{-1} DW). However, in field-grown shoot only trace amount of withanolide A was detected. Field-grown shoots (2.29 mg g^{-1}) had the highest withanone content as compared to *in vitro* C5 shoot (1.027 mg g^{-1}) and control *in vitro* shoot (1.3 mg g^{-1}) (Fig. 6).

4. Discussion

Heavy metal stress to *in vitro W. somnifera* shoots (without root) was studied to determine Pb responses in a plant without a root system. Among all the tested cultures, A3 of acute toxicity and C5 of chronic toxicity study had higher fresh biomass than other Pb-treated shoot cultures. In an acute toxicity study at higher concentrations (A4 and A5), shoots displayed heavy metal toxicity symptoms at longer treatment period (24–48 h), which is in agreement with the decrease of plant biomass and dark green colour leaves reported in *Elsholtzia argyi* by Islam et al. (2008), Islam et al. (2011) and by Khan et al. (2018) on *Brachiaria mutica* and *Ricinus communis*. In chronic toxicity study on exposure to higher concentration (C5) changes in leaf colour and width in response to Pb stress was observed which correlates with previous reports on *Avicennia marina* where leaf colour changed from green to dark green (Yan et al., 2010; Ali and Nas, 2018).

Among the five concentrations tested for chronic toxicity (C1-C5), biomass increase was positively correlated with the concentration and exposure period. Thus, biomass growth of *W. somnifera* shoots grown in suspension media supplemented with $PbAC_2$ highly

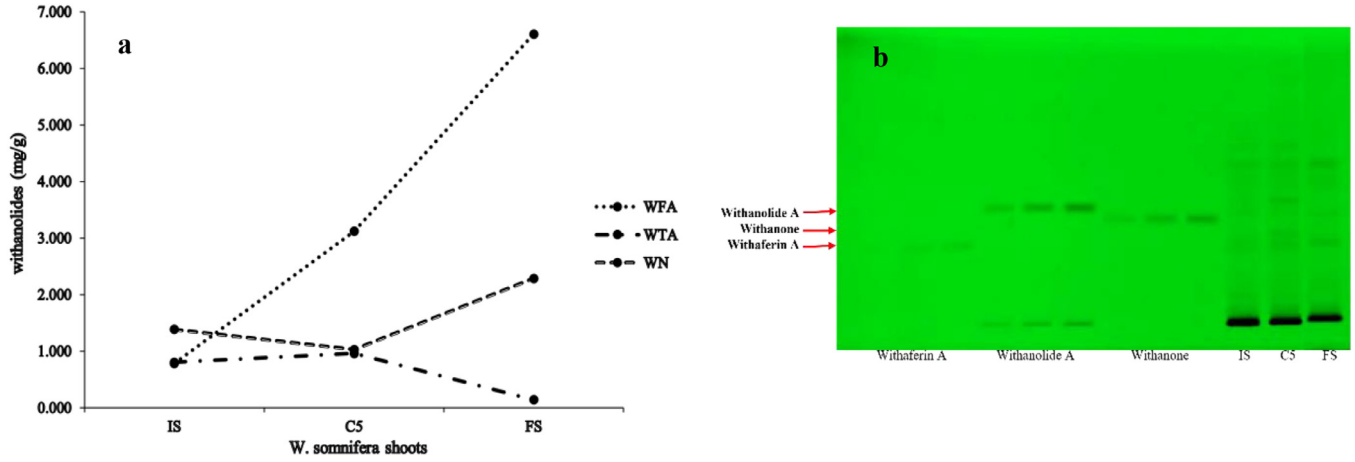

Fig. 5. Quantification of withanolides using HPTLC analysis. (a) Withanolides content (mg g-1 dry weight) in W. somnifera *in vitro* and field-grown shoots: WFA: Withaferin A; WTA: Withanolide A; WN: Withanone (IS- control *in vitro* shoot; C5 – *in vitro* Pb treated shoot; FS- field shoot). (b) HPTLC separations of standards and test samples of W. somnifera. Tracks 1–3 are of WFA (500–1500 μg), 4–6 are of WTA (1000–3000 μg), 7–9 are of WN (500–1500 μg) standards, and 10–12 represent control (IS), C5, and FS.

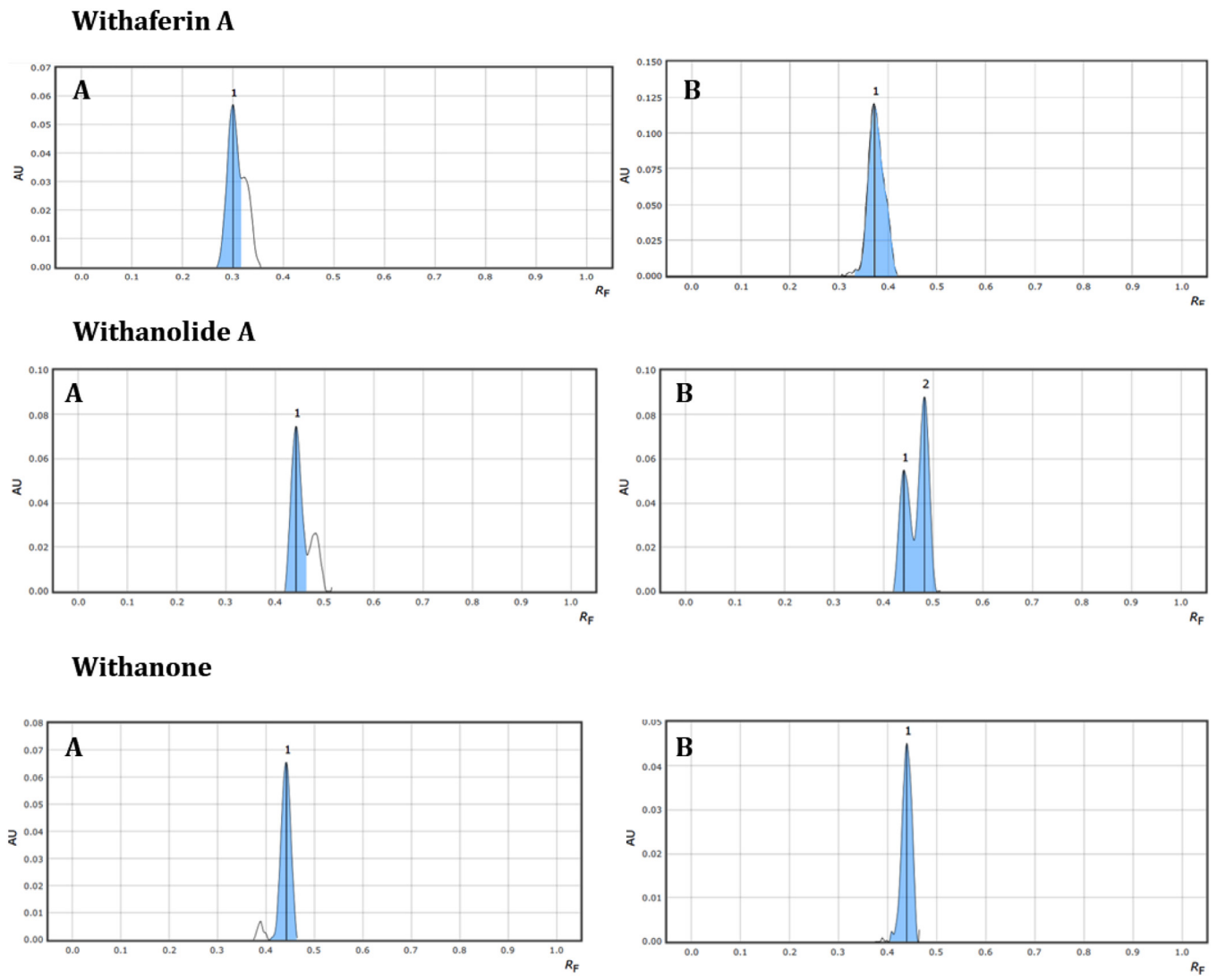

Fig. 6. Chromatogram of withaferin A, withanolide A & withanone in *in vitro* control (A) and Pb 800 μM treated (B) shoots of *W. somnifera*.

depends on the salt concentration and culture time. This is in line with a study by Deepthi and Satheeshkumar (2016), where cell growth of *Ophiorrhiza mungos* in cell suspension media was highly dependent on the elicitor concentration, incubation time, and feeding time of the elicitors (yeast extract and silver). Thus, the current results indicate that biomass (fresh weight) significantly correlates with long exposure to lower Pb concentration. This result was in agreement with (Paliwal et al., 2014), where they observed that *Helianthus annuus* plants exposed to Pb had a higher growth pattern at lower Pb concentration but a lower growth pattern at higher Pb concentration.

The leaves play a vital role in plant health and development, influencing metabolite production in response to stress conditions and high reduction potential towards the accumulated metal (Beattie and Haverkamp, 2011). According to Kulhari et al. (2013), *W. somnifera* presented higher metal bioaccumulation. The maximum permissible limit (MPL) for heavy/trace metals is higher for medicinal plants (WHO guidelines 2007) compared to edible plants (Filippini et al., 2018). Our results showed all heavy/trace metals analysed were within permissible limits in all tissues analysed, except in Pb treated tissues (WHO guidelines, 2007). The current study represents that *W. somnifera* shoot holds 40 times higher Pb content than the permissible limit for medicinal plants, yet the shoots were in good shape and healthy regardless of heavy metal exposure (Tripathi et al., 2014). This observation suggests the existence of tolerance mechanism, where, several other cations also get accumulated along with Pb. Here, during increase in Pb (heavy metal) exposure (C5), the K level increased from 21,109.36 mg kg^{-1} (control) to 24,779 mg kg^{-1} (C5) which shows the incomparable role of K in plant adapting to metal stress (Hasanuzzaman et al., 2018).

Calcium (Ca) was the second abundant element in the analysed sample. Calcium has several key functions: maintaining salt balance in plant cells, increasing nutrient uptake, protecting against plant ionic stress, etc. (Hasanuzzaman et al., 2018). The increase in Ca concentration in tissues exposed to Pb from 1411 (control) to 1787 mg kg^{-1} (C5) may be due to these functions. It was also found that the Fe improved plant growth, photosynthesis pigment, and photosynthesis during heavy metal stress (ul Hassan et al., 2017), which correlated with the higher biomass and darkened leaves observed during Pb stress. A study on Pb accumulation in *Typha latifolia* roots confirms that Fe can alleviate Pb toxicity in plants (Feng et al., 2013), which is in line with our results, where a healthy shoot appearance was observed during Pb chronic toxicity study. Further, it was found that Pb induced a strong reduction in Magnesium (Mg) content. The same result was found in pea plants stressed with cadmium which induced a strong reduction in Mg concentration (Rodriguez-Serrano et al., 2009). In conclusion, various studies revealed that K, Ca, & Fe alleviates heavy metal stress in plants (Tripathi et al.,

2014; ul Hassan et al., 2017); in return, the concentration of those metals was increased during the process, which was also observed in our studies (Table 1).

The distribution and accumulation of Pb as nanoparticles was observed in the leaves of *W. somnifera* C5 shoot. The electron micrographs observed the synthesized nanoparticles as spherical and rod-shaped structures in the form of single/clusters of different sizes. The size of nanoparticles ranging from 25 to 40 nm. The fact that nanoparticles can be formed and stored in plant tissues agrees with the report of Marchiol et al. (2014), who suggested that live plants of *Brassica juncea, Festuca rubra* and *Medicago sativa* can store Ag metal ions as silver nanoparticles after treatment with $AgNO_3$ metal salt. Further, the root tissues of Medicago, Brassica, and Fetusca species were capable of absorbing the metals via roots, transporting through xylem/phloem vessels, and accumulating them in the shoot and leaf part of the plant (Marchiol et al., 2014).

In plants, metals were absorbed and translocated as metal ions, and the nanoparticles seemed to form inside plant tissues. However, the exact mechanism for nanoparticle synthesis within the plant tissues is still largely unknown (Marchiol, 2012). However, according to previous reports, the phytoconstituents (Karmous et al., 2022) present in *W. somnifera* shoots might have acted as both a reducing and stabilizing agent, which results in the formation of PbNPs. EDAX spectrum shows the position of various elemental peaks in control and C5 samples. More specifically, the features at the following energies- 2.4, 2.45, 2.7 keV - are present in C5 samples alone. The energy peak at 2.4 & 2.45 keV in C5 samples confirms that Pb is in a reduced/metallic form. This is in line with Diba et al. (2021), where the PbNPs were synthesized from two species of bacteria, *Bacillus* sp. A21 and *Oceano bacillus* sp. A22 and EDAX spectrum of the PbNPs shows that the Pb peaks were at 2.4 & 2.45 keV energy levels. In another study by Miri et al. (2018), the synthesized PbONPs peaks are present at ~2.45 keV. The biological applications and biocompatibility of synthesized PbNPs/PbONPs using green route was reported by various researchers (Miri at al. 2018; Muhammad et al., 2019; Tailor and Lawal, 2021). According to the reports, PbNPs/PbONPs has higher antioxidant potential, antibiotics for the treatment of infectious diseases (Tailor and Lawal 2021), free radical scavenging activities (Muhammad et al., 2019), and even reported to have anti-cancer and anti-diabetic activities (Miri at al. 2018; Muhammad et al., 2019; Bratovcic 2020).

According to ICP MS results, a higher concentration of Ca (1787 mg kg^{-1}), K (24,779 mg kg^{-1}), and Fe (82 mg $kg^{-}1$) were found in C5 shoots than in control shoots. The same result was observed in the EDAX spectrum of our results, where the peaks of Ca, K & Fe were stronger in C5 shoots than in control shoots at 3.7, 6.4 & 3.4 keV, respectively. During heavy metal stress, the translocation of macronutrients like Ca, K, and Fe was increased to protect plants from heavy metal stress, which was observed in *Brassia campestris* L., *Vicia faba,* and *Prunus dulcis* seedlings, respectively (Tripathi et al., 2014; ul Hassan et al., 2017; Hasanuzzaman et al., 2018; Chen et al., 2019). Due to high levels of Ca, K, & Fe in leaves, strong peaks were observed in the EDAX spectrum of C5 shoots. These metals may be a part of MNPs' surface functional groups or present as individual elements in the leaf's cell-lysed solution of the analysed sample. Pb accumulates differentially among plant parts, especially in shoots. In general, metals translocated to shoots and accumulated in the above-ground organs, mostly leaves (Balafrej et al., 2020). Considering *W. somnifera* is a hyperaccumulator, it sequesters and accumulates metals in shoots, unlike plant excluders, which store the metals in roots (Seregin et al., 2014). Various response mechanisms enable the plant to withstand heavy metal toxicity, of which metal uptake and avoidance are the most common mechanism studied. The report shows that Pb mainly precipitates in the root cell wall, and only the free ions are transported to the other parts of the plant via xylem and phloem cells (Usman et al., 2020). Therefore, *in vitro* liquid medium facilitates the full availability of Pb ions (Jan and Parray, 2016) to the shoot cultures which is absorbed and accumulated as nanoparticles by the shoot cultures devoid of root tissues in the present study **(**Fig. 3**)** (Parker et al., 2014).

In the present study, $PbAC_2$ exposed *in vitro* shoot cultures of *W. somnifera* were subjected to withanolides quantification. Cai et al. (2017) reported that the metal treatment ($CaCl_2$ and $AgNO_3$) had little effect on furanocoumarin production in *Changium smyrnioides* suspension cells, which coincided with the decreased production of withanone during Pb treatment of this study. This study confirms that the production of different withanolides is responsive to heavy metal (Pb), and is concentration specific. The same trend was observed by Huang et al. (2021), where Methyl jasmonic acid (MeJA) proved to be the best elicitor for sanguinarine and chelerythrine production in *Macleaya cordata* cultures among different (MeJA, salicylic acid, and wounding) elicitors tested. Further, this study demonstrates that increase in withanolides levels is independent of biomass. Our results are in accordance with the results reported in *Phoenix dactylifera, O. mungos, A. marina* plants which have undergone biotic and abiotic stresses (Yan et al., 2010; Deepthi and Satheeshkumar 2016; Al-Khayri and Naik 2020).

Generally, Pb is immobile in the soil, and its extraction rate is limited by solubility and diffusion rate to the surface of plant root (Kushwaha et al., 2018). Thereby, Pb treatment via liquid medium in the presence of other metal salts increases the solubility and adsorption rate of Pb and presence of BAP (growth regulator) as a chelating agent helps Pb translocation from adsorption site (callus) to aerial part (shoot and leaf) (Israr and Sahi, 2008). The synergistic effect of plant growth regulators and heavy metal translocation was detailed by Mir et al. (2022) in *Brassica juncea*. Further, presence of growth regulators in the medium helps plant Camellia and Brasssica species to ameliorate heavy metal toxicity, enhanced plant growth and membrane stability (Xu et al., 2016; Mir et al., 2022). Studies have reported that, uptake and translocation of Pb may follow either of the following mechanisms. 1) Endocytic pathway and passive transport in to root cells then travels along xylem following water movement (Blanco et al., 2021). 2) Cation transporters including non-selective cations channels such as voltage-insensitive cation channels and depolarization/hyperpolarization -activated calcium channels are reported to be the principal route for Pb ions entry point into plant cells (Kushwaha et al. 2018). 3) Heavy metal transporters namely, ATPases-HMAs, zinc/iron-regulated transporter protein-ZIP and lipid transfer at contact site1-LTC1. Especially, HMAs strongly enhance the root-to-shoot translocation, which acts as a driving force for the hyperaccumulation in plants (Rascio and Navari-Izzo 2011). Aside from these finding, number of studies have reported other possible routes for Pb entry into plants (Wojas et al. 2007; Das et al., 2011) and also translocation of Pb ions within plants may vary depending upon the type of plant species (Kushwaha et al., 2018).

Several studies have reported that the most abundant sites for accumulation of absorbed metals as nanoparticles is in the green regions (leaf and stem) of the plant. The sites of MNP's accumulation within the cells maybe chloroplast (Beattie and Haverkamp 2011), cell wall (Marchiol et al., 2014; Schwab et al., 2016), plasmalemma (Marchiol et al., 2014), apoplast (Parker et al., 2014) and vacuoles of the mesophyll cells (Skuza et al., 2022) of the leaves. However, in our study, we were not able to observe the site of accumulation but nanoparticle formation was confirmed.

The hyperaccumulator plants are mainly used for phytoremediation (removal of heavy metal/ other contaminants from soil) and phytomining (growing the plant to harvest the precious metals) (Leitenmaier and Kupper 2013). In general, we report that plants have a capability of reducing accumulated metals (Pb) into nanoparticles in their native tissues using their own metabolites as a reducing and stabilizing agent (Marchiol et al., 2014). In the plant kingdom, 721 hyperaccumulating plant species have been reported till now

(Reeves et al., 2018). Among them, already reported medicinal plants with hyperaccumulating capability might have a higher metal reduction property to synthesize MNPs within their tissue. Therefore, with proper amendments *W. somnifera* can be used as bionano-factory to synthesize nanoparticles in higher quantity or nanoparticles with a desirable size and biological function which may have a synergistic activity with the increased secondary metabolites. Hence, the current study portrays cost effective, fast and stable formation of PbNPs which may be applicable in the industrial and medical sectors (Khan et al., 2023).

Collectively, we report that *in vitro W. somnifera* shoots exhibited tolerance to Pb, increased biomass and accumulating high secondary metabolites under Pb treatment. Additionally, we report that the enhanced reduction capability of *W. somnifera* shoots towards bioaccumulated Pb ions resulted in the *in planta* formation of PbNPs. With the available literature knowledge, this is the first ever report of *in planta* synthesis of PbNPs using *in vitro* shoot cultures of *W. somnifera*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2024.05.022.

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In planta synthesis of silver nanoparticles and its effect on adventitious shoot growth and withanolide production in *Withania somnifera* (L.) Dunal

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ABSTRACT

Silver (Ag) is a non-essential heavy metal with substantial environmental toxicity but an excellent promotor for plant organogenesis. It is used as an elicitor for secondary metabolite production and for *in planta* synthesis of metal nanoparticles (MNPs). In the present study, the Ag accumulation and reduction capability of *in vitro* shoots of *Withania somnifera* and the toxicity and elicitation effect of Ag on *in vitro* shoots were explored. *In vitro* shoot cultures of *W. somnifera* were treated with different concentrations of silver nitrate for a specific treatment period. Growth index, withaferin A, elemental and electron microscopy analyses were done on silver-treated *in vitro* shoots of *W. somnifera*. 1 mM silver nitrate treatment for 12 days period was found to give increased growth index (1.425 ± 0.05c) and withaferin A (2.568 ± 0.08e mg g^{-1}) content. The concentration of bioaccumulated Ag in 1 mM silver nitrate treated *in vitro* shoot was found to be 50.8 ppm. The presence of nano-Ag was also found in the leaves of 1 mM silver nitrate-treated *in vitro* shoots. In summary, this is the first report portraying the bioaccumulation and *in planta* reduction capability of the *in vitro* shoot system of *W. somnifera,* which makes it a potential medicinal plant of commercial value for silver contaminated soils.

1. Introduction

The practice of using plant/tree species to extract specific elements, particularly precious/pharmacologically important metals, from contaminated soils/aqueous waste is considered to be an effective way of cleaning up the environment. The selection of plant species for remediation purposes tends to meet certain criteria, which include availability, adaptability, socioeconomic importance, ethnobotanical background, and biocompatibility. However, the fate of metals in plants needs to be studied extensively in order to exploit the benefits of the system. Silver (Ag), a highly reactive and reducible transition heavy metal, is proven to be a non-toxic inorganic antibacterial agent (Bhuyar et al., 2020). Silver salt solutions exposed to sunlight or a simple reducing compound, i.e., proteins/sugars/metabolites, results in the synthesis of nanoparticles (NPs) (Bhaduri et al., 2013). The use of a particular synthesizing method and a type of biological medium determines the size and shape of NPs (Díaz-Núñez et al., 2017). Due to their limitless biological activity, Ag NPs are one of the most studied and synthesized nanoparticles using the green synthesis method, among which in planta synthesis of NPs is an alternative greener route where live plants/tissues can be used to fabricate metal nanoparticles (MNPs) (Jain et al., 2021; Esmail et al., 2022).

Withania somnifera is an Indian medicinal plant from the Solanaceae family that has heavy metal accumulation capability, high reduction potential, and enormous pharmaceutical applications. Due to its adaptogenic properties, it is used in hundreds of Ayurvedic formulations (Tetali et al., 2021; Natarajan et al., 2022). Withanolides, a group of secondary metabolites are confined to plants of the Solanaceae family, among which *W. somnifera* has a higher proportion of withanolides (steroidal lactones) (Chen et al., 2011). To date, more than 35 withanolides have been identified, and among them, withaferin A (WFA), withanolide A, and withanone are of significant pharmacological

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importance. WFA is considered to be therapeutically potential than any other withanolides and is reported to have anticancer, immuno-modulatory, anti-microbial, anti-diabetic, and stress-relieving activities (Vanden Berghe et al., 2012; Natarajan et al., 2022).

Field root and leaves of *W. somnifera* are included in herbal formulations in traditional systems of medicine (Ven Murthy et al., 2010); nonetheless, there is a chance for more foreign/cross contaminants and high heavy metal risks. Therefore, *in vitro* cultured tissues are being welcomed by most countries. From our previous results, we inferred that the secondary metabolite composition between *in vitro* shoot (IVS) is more similar to field-grown root of *W. somnifera* (FGR) (Senthil et al., 2015; Purushotham et al., 2017). We also found that field grown shoots (FGS) of *W. somnifera* has higher concentration of WFA, which was found to be toxic to human macrophage cells such as BMDM and raw 264.7 (Purushotham et al., 2017; Vinod et al., 2022). Although WFA was toxic in higher concentrations, a minor quantity is needed for *W. somnifera*'s pharmaceutical properties (Purushotham et al., 2017). Furthermore, the amount of WFA in FGR and IVS are nearly equal. In addition, the metal bioaccumulation was found to be higher in the shoot rather than root tissues of *W. somnifera* (Khan et al., 2007). Based on these observations, we have selected only shoot cultures for the metal elicitation study to determine the influence of Ag as an elicitor on *in vitro* shoot derived withanolides production.

The impact of nanomaterials in the environment on plant metabolism and homeostasis and the elicitation effect of MNPs on *in vitro* plants/tissues have been reported in detail (Iram et al., 2014). The synthesis of MNPs has increased due to their vast applications in almost all sectors (medical and industrial sectors). In addition, green synthesized MNPs (gMNPs) are reported to be less toxic to humans. But their turnover rate in the human body and the environment remains unclear (Jain et al., 2021). Compared to MNPs synthesized using toxic chemicals with toxic byproducts, green synthesized MNPs exert less toxicity in humans and the environment (Muhammad et al., 2019). There are reports available separately on *in planta* synthesis of MNPs, the effect of gMNPs on plant growth and metabolism, and the hyper accumulation of metal ions from soil/media to the plant tissues. However, the present study discusses the effect of metal ions and *in planta* synthesized gMNPs on plant growth and its impact on secondary metabolite production. Earlier studies have discussed the elicitation of withanolides in cell suspension cultures of *W. somnifera* using metals such as Cd, Cu, Ca, Al etc. (Baldi et al., 2008; Sivanandhan et al., 2014; Siddiqui et al., 2015; Mishra and Singh, 2019), in whole plant cultures using Fe, Zn, and Cu (Rout et al., 2013, 2015, 2019) and in adventitious root cultures in presence of Al (Sivanandhan et al., 2012). Further, most toxicological studies were conducted using acute toxicity (short-term exposure to high dose), but the environmental effects can be more effectively assessed by chronic toxicity testing (long-term exposure to low dose) (Kaveh et al., 2013).

Taking this into consideration, the objectives of this study were: (i) examine the effect of $AgNO_3$ on IVS of *W. somnifera* under acute and chronic conditions; (ii) analyse the ability of $AgNO_3$ to elicit withanolides production, and (iii) characterize the bioaccumulated form of Ag at in planta level.

2. Materials and methods

2.1. Plant genetic materials

W. somnifera Jawahar 20 (JA20) seeds were obtained from the Indian Council of Agricultural Research- Central Tobacco Research Institute (ICAR - CTRI), Rajahmundry, India. The seeds were soaked overnight and sterilized using Sodium hypochlorite (2.8%) with few drops of Tween 20 solution. The primary shoot culture was grown in solid half-strength Murashige and Skoog (MS) basal medium followed by full-strength MS basal medium. The one-month-old MS-grown shoots were subcultured onto MS supplemented with 4.44 μM 6 benzyl aminopurine medium (BAP medium) for shoot multiplication. All shoot cultures were kept in 16/8 h photoperiod (25 μmol m^{-2} s^{-1}) at 25±2 °C. All the plant tissue culture experiments were done in Avinashilingam Institute, Coimbatore, India.

2.2. Establishment of shoot suspension culture and silver nitrate treatment

One-month-old BAP-grown shoots were transferred to a liquid BAP medium (suspension medium without agar) to increase shoot biomass. The liquid cultures were grown under fluorescence on a rotary shaker at 50 rpm and 25±2 °C for 15 days. At the end of the 15th day, 45 days old healthy shoots with equivalent weight were selected for $AgNO_3$ treatment. Initial weight was calculated, and shoots were inoculated onto liquid BAP medium supplemented with different concentrations of $AgNO_3$ solution. For acute toxicity studies, the shoots were exposed to a higher $AgNO_3$ concentration (1–16 mM) (200–3200 ppm) and a shorter duration (12–48 h), whereas for chronic exposure, a lower $AgNO_3$ concentration of 0.25–4 mM (50–800 ppm) and a longer duration (4–12 days) were chosen. Following the treatment period, shoots were harvested, and the growth index (GI) was calculated [GI= (Harvested fresh weight-inoculated fresh weight/inoculated fresh weight) * 100]. The Ag concentration was chosen based on the Ag concentration in the Indian contaminated and non-contaminated soils ranging from 10 to 7000 ppm (Kolesnikov et al., 2020). For metal salt treatment, the stock solutions were prepared by dissolving $AgNO_3$ in heat-sterilized double distilled water and kept in the dark until treatment. The selected concentration of $AgNO_3$ for acute and chronic toxicity studies was added to the media from the stock solutions.

2.3. HPTLC profiling of withanolides

For HPTLC analysis, *in vitro* and field-grown *W. somnifera* tissues were shade-dried and extracted using methanol. 0.5g of dried tissue powder and 100% methanol was used for the extraction. The extraction procedure was followed according to Senthil et al. (2015). The resultant concentrated methanolic extract was undergone withanolide quantification. The protocol reported by Vinod et al. (2022) was followed for HPTLC analysis (CAMAG). Briefly, silica gel coated TLC aluminum sheets (60 F_{254}, Merck, Germany) were used as a stationary phase, and a freshly prepared toluene, ethyl acetate, and formic acid in the ratio of 5:5:1 v/v solution was used as a mobile phase. The standards withaferin A, withanone, and withanolide A peaks were used as reference peaks and compared to the sample peaks to quantify different withanolides in *in vitro* and field-grown shoots of *W. somnifera*.

2.4. Tissue processing and electron microscopy analysis for in planta-synthesized nanoparticles

Ultra-structural analysis was performed by bright field transmission electron microscopy (TEM) (FEI, Tecnai Sprit G2, Netherlands). For TEM analysis, the protocol reported by Starnes et al. (2010) was followed with slight modifications. Briefly, 50 mg fresh young leaves (2nd leaf from shoot apical meristem) were excised carefully and washed three times in HPLC grade water. The leaf tissues were cryo-grounded and suspended with 2 ml HPLC-grade water. A 0.5 ml aliquot of the shoot extract was pulse-sonicated on ice (10s at 95% amplitude followed by 15s rest with 5 pulses per cycle) with 3 mm standard probe of the VCX500 Ultrasonic Processor with anti-noise cabinet (Sonics & Materials). The probe temperature was maintained at 20 °C. After each sonication cycle, the shoot extract was centrifuged at 4 °C for 30s at 10, 000 rpm in a table-top centrifuge (Rota R–V/Fm, Plasto Crafts). The sonication-centrifugation cycle was repeated three times to increase the tissue lysis. At the final cycle, the sonicated extract was pelleted by centrifugation, and 10 μL was transferred onto the 400-mesh copper-coated grids. Point and region analyses were performed at 120 kV. The EDAX spectra with silver (Ag) peaks were recorded, and

micrographs were captured at 40 keV for 30–40s.

2.5. Elemental analysis by inductively coupled plasma mass spectrometry

Elemental analysis on *in vitro* and field-grown tissues of *W. somnifera* was done by ICP MS (Thermo fisher iCAP RQ ICPMS). The essential trace elements, essential and non-essential heavy metals for plants, were quantified using the manufacturer's guidelines. Briefly, dried *W. somnifera* tissue was powdered using mortar and pestle, and 0.2g was taken and digested using 6.0 ml of HNO3 (67–70% concentration) in Anton Paar Multiwave GO microwave digestor for 30 min at 453K. The experimental solution was made up to 50 ml in a volumetric flask, and the solution without plant sample served as blank.

2.6. Statistical analysis

Data on the effect of $AgNO_3$ on shoot growth index and withaferin A in control and treated shoots were analysed and expressed as mean ± standard error of the mean. Duncans multiple range test (DMRT) was done to separate the mean values ($p \leq 0.05$) using computer-aided software SPSS (ver. 16.0).

3. Results and discussion

3.1. Influence of silver on biomass accumulation

In vitro shoots of *W. somnifera* undergone $AgNO_3$ treatment, and among the test and control cultures, 1 mM Ag treatment (E1D3) for 12 days (T6) emerged as a promising dose to induce higher biomass (17.24 g) (Data has not shown) and growth index (GI) (1.425 ± 0.05) (Table 1). During $AgNO_3$ treatment, noticeably, the shoots showed two distinctive coloring patterns (pale green and dark brown) at both acute and chronic conditions. The blackening/browning of calli was observed at high concentrations of $AgNO_3$ treatment, possibly due to Ag oxidation, and oxidized Ag may be in contact with media components and/or calli. Further, blackening of the calli affects the shoot proliferation and GI rate, which was found at >1 mM $AgNO_3$ concentration at all exposure periods. In our study, shoots were harvested after acute and chronic treatment, and the GI was calculated. The shoot growth rate and shoot morphology were altered during $AgNO_3$ treatment (Table 1 & Fig. 1). In acute treatment, the higher concentration of Ag negatively affected shoot biomass accumulation even at shorter duration (T2 & T3). Due to its reactive properties, Ag caused cell/calli browning, edema and, at higher concentrations (E1H4 & E1H5), the shoots blackened and died within 48 h (Supplementary Fig. 1). Similar cell browning and cell death was reported in *Ophiorrhiza mungos* cell suspension cultures treated with different concentrations of $AgNO_3$ (2–7.5 μM) to elicit camptothecin content (Deepthi and Satheeshkumar 2016). In a study on *Eruca sativa* seeds exposed to $AgNO_3$, the seedling's growth was increased up to the concentration of 10 mg/l; however, further, increase in $AgNO_3$ concentration to 20 mg/l completely blocked the germination (Vannini et al., 2013). Similarly, many reports show that the secondary metabolite accumulation pattern varies depending on the type of elicitors and a specific treatment period (Karwasara and Dixit, 2013; Ahmed and Baig, 2014; Deepthi and Satheeshkumar 2016).

Among acute treated shoots (higher concentration of $AgNO_3$ to shorter duration), H2 (2 mM) exhibited increased GI compared to other concentrations (H1, H3–H5). Higher concentrations of $AgNO_3$ treatment (H4 & H5) reduced GI compared to control and other acute treated shoots' GI. A similar study showing an increase in biomass in response to Ag concentration is reported by a few researchers, in which, at higher Ag concentration, the plant biomass was significantly reduced (Jiang et al., 2012). On low concentration of Ag treatment for a longer duration, shoots' GI was increased up to E1D3T6 (1 mM), which showed maximum growth index (1.425). Further, an increase in the Ag concentration (E1D4 & E1D5) at all durations (T4-T6) was favourable for shoot growth (Fig. 1). However, GI was decreased significantly (Table 1). Compared to control shoots (0.314), a 4.54-fold increase in GI was observed at E1D3 (1 mM) (1.425), which served as a rate-limiting concentration for IVS of *W. somnifera*. In addition, our results corroborate the studies of Al-Khayri and Naik (2020) where the date palms suspension cultures treated with different elicitors [pectin, yeast extract, salicylic acid, cadmium chloride, and silver nitrate] at lower concentrations showed a positive impact on biomass accumulation. However, elicitors at higher concentrations suppressed cell growth significantly. A study on *Ocimum basilicum* reported that a rise in seed yield was observed when treated with a higher concentration of $AgNO_3$ up to 0.6 mM compared to the control (Nejatzadeh-Barandozi et al., 2014). Similar effects of silver salts in *Arabidopsis thaliana* seedlings have also been reported previously by (Wang et al., 2013).

In the present study, we used 45-day-old IVS for metal (Ag) toxicity studies, and the type of plant species we have chosen is a plant with hyperaccumulator capability. These might be the reason why the shoots were healthy (Fig. 2) even at high $AgNO_3$ (1 mM) concentration and duration (12 days) (E1D3T6), which is higher than the aforementioned report (Al-Khayri and Naik, 2020). Further, the presence of BAP in the medium as a chelating agent helps metal (Ag) adsorption and translocation within the plants (Israr and Sahi, 2008). Thus, E1D3T6 was selected as the optimum dosage for IVS of *W. somnifera* and taken up for further studies.

3.2. Toxicity of Ag towards in vitro shoot cultures of W. somnifera

Due to higher concentrations of $AgNO_3$ in acute exposure conditions (E1H3-E1H5), a change in colour of the basal shoot (with callus) from pale green to brownish black (reduced Ag^+) along with yellowing of leaves was observed (>4 mM) at all treatment period (T1-T3) (Supplementary Fig. 1) and reduction in GI as well (Table 1). This weight decline may be due to the excess availability of Ag^+ that disrupted the cellular function, triggering cytotoxicity and growth inhibition in plants (Jiravova et al., 2016; Açıkgöz, 2020). Similarly, cytotoxicity response due to increased oxidative stress on shoot growth was reported in several previous investigations (Panda et al., 2011; Kaveh et al., 2013). Yellowing of leaves was observed here may be due to heavy metal stress (Ag), which was reported by (Feng et al., 2023). Our results corroborate the studies of Al-Khayri and colleagues, where the date palm suspension

Table 1
Growth index of *in vitro* shoot cultures of *W. somnifera* exposed to $AgNO_3$ at different concentrations and duration.

Acute treatment	T1	T2	T3	Chronic treatment	T4	T5	T6
Control	0.0160 ± 0.004^{b}	0.0603 ± 0.04^{e}	0.1196 ± 0.02^{e}	Control	0.1607 ± 0.003^{a}	0.23 ± 0.003^{a}	0.3143 ± 0.002^{a}
E1H1	0.0121 ± 0.002^{b}	0.0431 ± 0.005^{d}	0.0692 ± 0.003^{d}	E1D1	0.2061 ± 0.005^{b}	0.2658 ± 0.01^{a}	1.009 ± 0.05^{b}
E1H2	0.0432 ± 0.005^{d}	0.0605 ± 0.004^{e}	0.0930 ± 0.005^{de}	E1D2	0.2683 ± 0.01^{c}	0.3920 ± 0.01^{c}	1.3037 ± 0.07^{c}
E1H3	0.0274 ± 0.001^{c}	0.0026 ± 0.001^{c}	0.0121 ± 0.003^{c}	E1D3	0.4465 ± 0.02^{e}	0.6088 ± 0.06^{d}	1.425 ± 0.05^{c}
E1H4	0.0108 ± 0.001^{b}	0	0	E1D4	0.3851 ± 0.001^{d}	0.3648 ± 0.01^{bc}	0.8876 ± 0.05^{b}
E1H5	0	0	0	E1D5	0.142 ± 0.03^{a}	0.2776 ± 0.01^{ab}	0.3626 ± 0.01^{a}

Note: T1-T6 indicates the different durations (12, 24 & 48 h, and 4,8 &12 days) of acute and chronic silver treatment. E1: AgNO3 treatment; H1–H5: 1mM–16mM AgNO3 acute treatmet and D1-D5: 0.25mM–4mM AgNO3 chronic treatment. Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at $p \leq 0.05$, according to DMRT. The mean difference is significant at the 0.05 level.

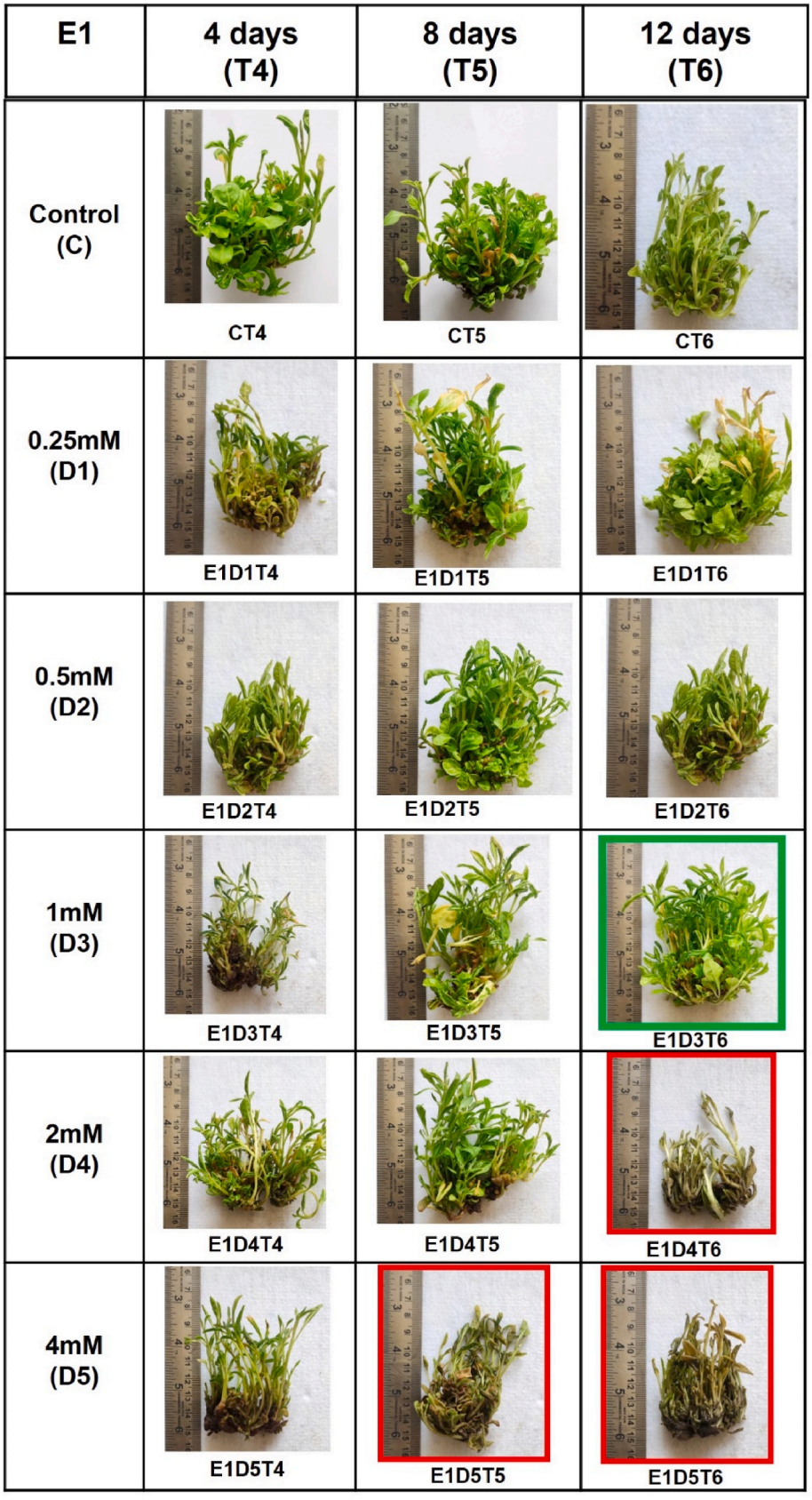

Fig. 1. The chronic treatment of silver nitrate on *in vitro* shoot cultures of *W. somnifera*.
Note; Highlighted picture in green represents maximum growth index with healthy shoots and picture in red represents drying and and blackening of shoots due to silver toxicity.

Fig. 2. (a) Control *in vitro* shoots (CT6) and **(b)** E1D3T6 (1 mM $AgNO_3$ treatment for 12 days) shoots with increased biomass.

cultures treated with $AgNO_3$ at lower concentrations positively impacted biomass accumulation, and higher concentrations suppressed cell growth significantly (Al-Khayri and Naik, 2020).

3.3. Silver nitrate treatment on withanolides production – in vitro

A study by Wink (2016) reported that, though secondary metabolites are not important for plant growth and development, they have an important function in the plant defense mechanism. The evolution of secondary metabolic pathways in plants is an adaptation mechanism developed due to environmental stressors (Wink, 2016). Based on the chemical composition, stressors are classified as biotic and abiotic stressors/elicitors. Upon entering, these elicitors tend to activate the production of secondary metabolites, protecting the plants against external stressors (Gonçalves et al., 2019). In the present study, all $AgNO_3$-treated IVS were harvested after a specific treatment period and subjected to HPTLC analysis for withaferin A (WFA) and other withanolides (withanolides A and withanone) quantification. Many studies have reported that Ag increased secondary metabolite content in plants and plant tissue cultures at a specific concentration and duration (Mahendran et al., 2018). The accumulation pattern of biomass and WFA was similar in both acute and chronic treated IVS, confirming that biomass accumulation was positively correlated with WFA production.

During higher concentration of $AgNO_3$ treatment for a short duration, significantly increased WFA production till E1H4 (8 mM) for 12 h treatment (T1) (0.828 mg g^{-1}). However, in the T2 & T3 period, E1H3 (4 mM) showed increased WFA rather than E1H4, showing the adaptive nature of IVS of *W. somnifera* to $AgNO_3$ treatment. Similar to our results, secondary metabolite accumulation patterns change depending upon the incubation period of specific elicitors in *Ophiorrhiza mungos* cell suspension cultures (Deepthi and Satheeshkumar, 2016; Mahendran et al., 2018). Thus, secondary metabolite production depends on elicitor concentration, type of plant species, and exposure time (Gonçalves et al., 2019; Açıkgöz, 2020). For instance, in a study on shoot cultures of *Salvia virgata* Jacq. treated with 2.5 ppm Ag ions resulted in increased rosmarinic acid accumulation after five days of treatment (Attaran Dowom et al., 2017); on the other hand, increased accumulation of rosmarinic acid was found at 72 h of treatment with 30 mg/ml $AgNO_3$ in *Agastache rugosa* cell cultures (Park et al., 2016). Further, a maximum of 1.10 mg g^{-1} dry wt. of WFA was noted in E1H3T2, and the least was in control (0.556 mg g^{-1}) followed by E1H1-E1H5 (T1) (1.2–1.6 mg g^{-1}) (Table 2). Likewise, in chronic exposure to $AgNO_3$, a maximum amount of WFA was found in E1D3 (2.57 mg g^{-1} DW) after 12 days of treatment (T6), and at higher doses (E1D4 & E1D5), WFA yield was significantly declined. In chronic studies, minimum WFA was observed in control (0.764 mg g^{-1} DW) (CT6) followed by E1D1T4-E1D4T4 (1.26–1.98 mg g^{-1}) (Table 2). WFA concentration increased significantly from E1D1-E1D4 of T4 and T5 chronic exposure but decreased at E1D4 of T6 and E1D5 of T6. WFA content in IVS was highest at E1D3T6 (2.57 mg g^{-1}) (Table 2). Similarly, the report on *Agastache rugosa* cell cultures treated with different concentration of $AgNO_3$ increased the amount of rosmarinic acid in a concentration-dependent manner (Park et al., 2016). Following WFA quantification, the accumulation pattern of different withanolides was studied in E1D3T6 shoots (optimum Ag treatment), CT6 (control IVS), and field-grown shoots (FGS) of *W. somnifera*. The accumulation of withanolides in E1D3T6 was found to be increased for WFA (2.54 mg g^{-1}) and WTA (0.600 mg g^{-1}) and decreased for WN (0.709 mg g^{-1}) compared to CT6 (Fig. 3). Same trend was observed in a study reported by Cai et al. (2017) where MeJA (methyl jasmonic acid) was proved to be the best elicitor for

Table 2
Silver treatment on the production of withaferin A in in vitro shoot cultures of *W. somnifera*.

Acute treatment	WFA (mg g^{-1} DW)			Chronic treatment	WFA (mg g^{-1} DW)		
	T1	T2	T3		T4	T5	T6
Control	0.556 ± 0.01^{a}	0.583 ± 0.02^{a}	0.599 ± 0.02^{a}	Control	0.669 ± 0.01^{a}	0.722 ± 0.01^{a}	0.764 ± 0.02^{a}
E1H1	0.620 ± 0.02^{ab}	0.616 ± 0.02^{a}	0.737 ± 0.01^{b}	E1D1	1.263 ± 0.02^{b}	1.429 ± 0.06^{b}	1.916 ± 0.03^{bc}
E1H2	0.642 ± 0.03^{ab}	0.925 ± 0.01^{c}	0.799 ± 0.01^{cd}	E1D2	1.599 ± 0.04^{c}	1.688 ± 0.09^{c}	2.297 ± 0.12^{d}
E1H3	0.680 ± 0.01^{b}	1.016 ± 0.01^{d}	1.097 ± 0.01^{e}	E1D3	1.776 ± 0.05^{d}	2.204 ± 0.09^{de}	2.568 ± 0.08^{e}
E1H4	0.828 ± 0.01^{c}	0.762 ± 0.03^{b}	0.826 ± 0.01^{d}	E1D4	1.982 ± 0.03^{e}	2.328 ± 0.09^{e}	2.107 ± 0.13^{cd}
E1H5	0.795 ± 0.04^{c}	0.567 ± 0.03^{a}	0.766 ± 0.01^{bc}	E1D5	1.204 ± 0.01^{b}	1.957 ± 0.09^{d}	1.731 ± 0.04^{b}

Note: T1-T6 indicates the different durations (12, 24 & 48 h, and 4,8 &12 days) of acute and chronic silver treatment. E1: AgNO3 treatment; H1–H5: 1mM–16mM AgNO3 acute treatmet and D1-D5: 0.25mM–4mM AgNO3 chronic treatment. Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at $p \leq 0.05$, according to DMRT. The mean difference is significant at the 0.05 level.

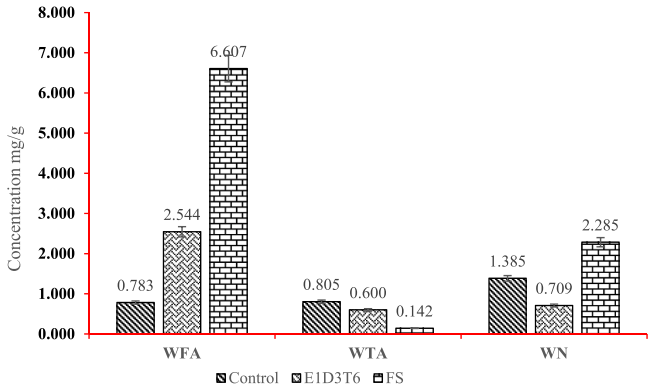

Fig. 3. Accumulation of three withanolides (WFA withaferin A; WTA: withanolide A; WN: withanone) in in vitro (Control and E1D3T6) and field-grown shoots of *W.somnifera*.

furanocoumarin production and $CaCl_2$ & $AgNO_3$ found to had very little effect on furanocoumarin production in *Changium smyrnioides* suspension cells. Many other studies detailed the elicitor-specific increment in the secondary metabolite production in plant cell/tissue cultures (Kisa et al., 2016; Cai et al., 2017; Huang et al., 2021). The concentration of WFA and WN levels is found to be higher in FGS than in E1D3T6 and control shoots, but WTA is at a negligible level in FGS of *W. somnifera*. Production of secondary metabolites in plants is greatly influenced by the type of elicitor used, feeding time of elicitor, duration of elicitation, and type of plant species. In addition, the type of culture conditions used for the elicitation, regardless of the type of elicitors used, may be useful in determining optimum growth conditions (Zafari et al., 2016; Park et al., 2016). The exact mechanism of these abiotic elicitor's action in promoting yield is not known yet, but it probably up-regulates genes encoding enzymes like sterol Δ-7 reductase or enzymes of the DOXP pathway which participate in withanogenesis (Agarwal et al., 2017). This was proved in the hairy root culture of *S. miltiorrhiza*, where the silver ions were found to stimulate the production of tanshinones, which can be dependent on both mevalonate and non mevalonate pathways due to the induction of key enzymes (Ge and Wu, 2005). Thus, the results obtained in the present experimentation indicate that abiotic elicitation could be a promising approach for triterpenoid (withanolides) enrichment. The process could also be utilized for large-scale production of pharmacologically important compounds, i.e., withanolides from IVS of *W. somnifera*.

3.4. Ultra-structural analysis of in vitro shoots of W. somnifera

Of primary interest in this present work is to determine the Ag reduction and nanoparticle accumulation capability of mature (45 days old) IVS (without root) of *W. somnifera* treated with optimum dosage (1 mM & 12 days) of $AgNO_3$ (E1D3T6). $AgNO_3$-treated live leaf mesophyll cells were cryo-grounded, lysed with sonication, and centrifuged to separate the cell debris from internal cell components (including minerals and nanomaterials). The supernatant part was analysed for the presence of nanoparticles. The plants have rich sources of reducing compounds and capping agents. Plants usually uptake the metal in its ionic form, and absorbed metal ions are transported through metal transporters/ion exchangers to the greener regions of plants. Further, various studies have reported that these absorbed metals might get reduced to nanoparticles on their way to their final destination (cell organelles) or nanoparticles formed outside the plant and then translocated into the root tissues (Haverkamp and Marshall, 2009). In contrast with these reports, there have been debates on metal ion absorption, reduction, bioaccumulation, and transportation of metals as MNPs within the plants (Usman et al., 2020). However, few studies have reported that metals were taken up by root cells as ions and reduced within the plant into nanoparticles (Marchiol et al., 2014). Similarly, the metal reduction rate within a plant also depends on the reduction and bioaccumulation potential of the host plant.

In the current study, electron microscope analysis on the tissue lysates revealed the presence of nanostructures displaying spherical and rod shapes. The same trend was observed in *Medicago sativa*, *Brassica juncea*, *Fetusca rubra,* and *Sesbania drummondii* plant seedlings exposed to different concentrations of Ag/Au which resulted in the accumulation of Ag/Au NPs in their tissues (Sharma et al., 2007; Harris and Bali, 2008; Beattie and Haverkamp, 2011; Marchiol et al., 2014; Bhaskaran et al., 2019). Live plant's accumulation and reduction capability to synthesize gold and silver nanoparticles was first reported in 2002 (Gardea--Torresdey et al., 2002, 2003). In the present study, the control shoot (CT6) is void of any nanostructures (Supplementary Fig. 2), and the EDAX spectrum of CT6 shows the presence of all essential nutrients, and no silver was found (Fig. 4). On the other hand, the analysed silver treated sample (E1D3T6) was observed to have nanomaterials size ranging from 20 to 40 nm (Fig. 5). The nature of the accumulated Ag in the shoots was confirmed as nanoparticles, and the nanostructures present in the sample were confirmed as metallic silver (Ag^0) using EDAX results. Ag NPs are most commonly synthesized MNPs using the green synthesis method. There are enormous reports on Ag NPs synthesis using plant extracts (Singh et al., 2018), microbial biomass/extracts (Bhuyar et al., 2020), individual components (phytoconstituents) (Raveendran et al., 2003; Ayaz Ahmed et al., 2014; Iram et al., 2014) or even using sunlight as a reagent (Bhaduri et al., 2013; Tang et al., 2015). These studies show that Ag^0 was present in the EDAX spectrum at ~3 keV (Fig. 5) (Bhaduri et al., 2013; Bhuyar et al., 2020). The presence of Ag peak at 3 keV represents that Ag was reduced by phytoconstituents in *W. somnifera* shoots, which are probably withanolides. The results were comparable with Dauthal and Mukhopadhyay (2016), where the presence of phyto-constituent present in the plant extracts was utilized for the NP synthesis, and the process was called extra-cellular synthesis of nanoparticles (Dauthal and Mukhopadhyay, 2016). Further, point and region analysis by TEM-EDAX on the found nanostructures revealed the presence of a high proportion of Ag along with Cu (Cu grid). This shows that the found nanostructures were, in fact, Ag NPs.

Probable mechanisms for *in planta* synthesis of nanoparticles within plants were addressed in numerous studies (Beattie and Haverkamp, 2011; Marchiol et al., 2014). On the other hand, few studies reported that the exact machinery behind reducing specific metals in live plant systems could not be addressed without proper scientific data (Iravani, 2014; Parker et al., 2014). However, many researchers have reported

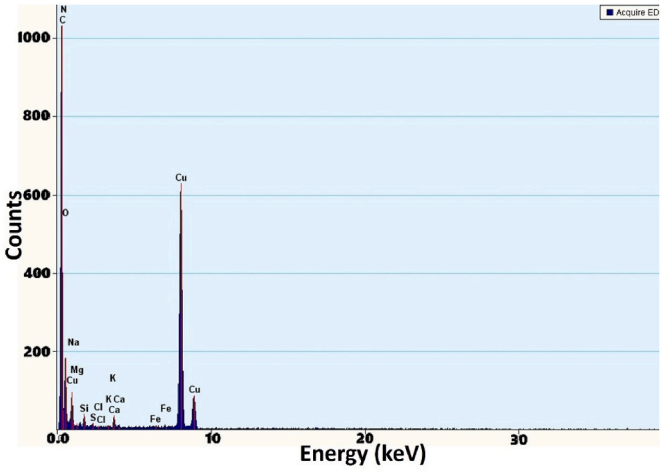

Fig. 4. Point and region analysis on TEM micrograph by EDAX on CT6 indicates the presence of essential metals for plant growth, such as Mn, Fe, Na, Mg, Ca, Zn, and Na in lower counts; however, no silver was found.

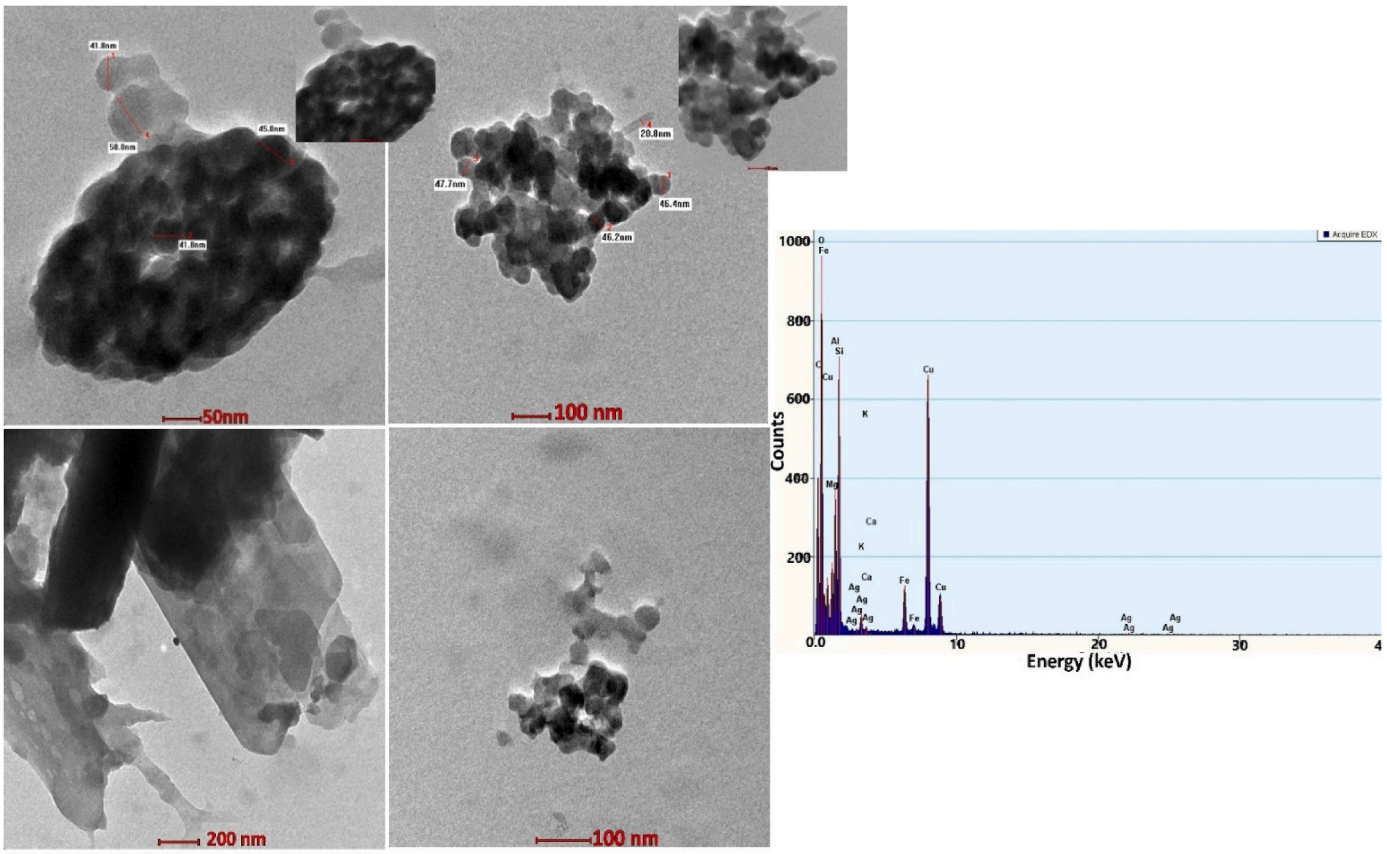

Fig. 5. (a) The presence of Ag NPs in live leaf fractions from *in vitro* shoot cultures of W. somnifera. The magnified picture of NPs represented within the figures **(b)** EDAX analysis indicates the presence of metallic silver at 3 keV energy levels. Essential metals for plant growth such as K, Ca & Fe levels increased in E1D3T6 shoots.

the exact mechanism of metal uptake by plant roots, transportation of metal ions within plant tissues, and reduction and accumulation of metal ions (Jiang and Liu, 2010; Ul Hassan et al., 2017). Further, the mechanism for *in planta* formation of NPs is metal ions and plant species-specific. Thus, proper scientific investigation is needed to understand the route for Ag ions and their reduction in *W. somnifera* IVS. In a study on Palladium nanoparticles (Pd NPs) synthesis using *Arabidopsis thaliana* seedlings via *in planta* route reported that the mechanism behind Pd NP synthesis could be a chemical rather than biological process, i.e., Suzuki-Miyaura reactions (Parker et al., 2014).

As for the transporation, the translocation of Ag NPs in plants is reported to be by both apoplastic and symplastic pathways. Further, numerous studies have been reported on the translocation and penetration (passive transport) of Ag NPs into plant cells, which accumulate inside vacuoles and other cell organelles (Larue et al., 2012). The uptake and translocation of Ag NPs are species-specific, and NP size-dependent, which was proved in tobacco seedlings where 3.5 nm Au NPs are present in shoots and 18 nm Au NPs are present in the root outer surface (Sabo-Attwood et al., 2012).

3.5. Elemental analysis on in vitro and field samples of W. somnifera

Twenty-three heavy and trace metals including Ag were analysed in silver elicitated shoots (E1D3T6) and non-elicitated dried *in vitro* (CT6) and filed tissues (FGS) of *W. somnifera*, and only 22 elements (except Sb) were detected in all tissues. Ag was not detected in non-elicitated IVS shoots (CT6). But FGS was found to have all heavy and trace metals detected within the permissible limit set by FAO/WHO (except for iron and copper) (WHO guidelines 2006). Among all tested samples, FGS had the highest concentration of trace metals in descending order of K, Ca, Mg, P, B, and Cu (Table 3). Increased concentration of Ag was found in E1D3T6 (50.8 mg g-1) shoots, followed by FGS (0.015 mg g-1). The percentage of Ag in E1D3T6 shoots was quantified as 2.54%. At 200 mg kg^{-1} Ag treatment for 12 days (E1D3T6), shoot Ag accumulation was

Table 3
Elemental composition of silver treated and control *in vitro* and field shoots of *W. somnifera*.

S. no.	Parameters	CT6 (ppm)	E1D3T6 (ppm)	FGS (ppm)
Essential metals for plant growth				
1	11B (Boron)	21.26	15.088	31.45
2	24 Mg (Magnesium)	1282.32	727.499	7895.97
3	31P (Phosphorus)	3679.81	1773.41	1941.089
4	39K (Potassium)	21,109.37	26,077.91	27,396.52
5	44Ca (Calcium)	1411.69	1833.64	8689.188
6	55Mn Manganese)	195.56	134.62	41.16
7	57Fe (Iron)	11.203	85.101	10.502
8	95Mo (Molybdenum)	2.803	1.763	0.3311
Essential heavy metals for plant growth				
1	60Ni (Nickel)	0.0032	0.066	0.9871
2	63Cu (Copper)	2.0273	0.385	8.1969
3	66Zn (Zinc)	42.2345	25.989	15.586
4	48 Ti (Titanium)	Nil	Nil	3.667
Heavy metals for plant growth				
1	52Cr (Chromium)	0.0076	0.0088	0.2076
2	59Co (Cobalt)	0.1452	0.141	0.1635
3	75 A s (Arsenic)	0.0045	0.005	0.0162
4	111 C d (Cadmium)	0.0055	0.003	0.0196
5	202 H g (Mercury)	0.004	Nil	0.0002
6	107 A g (Silver)	Nil	50.801	0.015
7	208 P b (Lead)	0.0279	Nil	0.1132
8	51V (Vanadium)	Nil	Nil	0.03
10	7 Li (Lithium)	Nil	Nil	0.026

Note; CT6: control *in vitro* shoots; E1D3T6: 1 mM AgNO3 treatment for 12 days period; FGS: field grown shoots of *W. somnifera*.

50.8 mg kg^{-1} dry weight of the tissue. Reports show that among field-grown tissues, leaves and shoots had the highest bioaccumulation potential than other tissues (Kulhari et al., 2013). The MPL (maximum permissible limit) for heavy/trace metals is higher for medicinal plants. According to European Pharmacopeia, the following MPL are available: 1 μg g^{-1} Cd, 2 μg g^{-1} Cr, 0.1 μg g^{-1} Hg, 5 μg g^{-1} Pb (Marinescu et al., 2020). The Chinese Pharmacopeia recommends 2 μg g^{-1} As, 0.3 μg g^{-1} Cd, 20 μg g^{-1} Cu, 0.2 μg g^{-1} Hg (Li et al., 2012) and WHO/FDA set the MPL: 10 μg g^{-1} As, 0.2 μg g^{-1} Cd, 20 μg g^{-1} Cu, 1 μg g^{-1} Hg, 10 μg g^{-1} Pb, 50 μg g^{-1} Zn (WHO guidelines 2007). Further, compared to CT6 (control), E1D3T6 was found to have increased concentrations of K, Ca, and Fe (Table 3). This increase in essential metal concentration during heavy metal stress on plants was also observed in many other studies (Tripathi et al., 2014; Karthika et al., 2018). The result represents that Ag in $AgNO_3$-treated IVS of *W. somnifera* was 50.8 mg kg^{-1}. Ag content in plant tissues usually ranges from 0.01 to 0.067 mg kg^{-1} (Hajar et al., 2014). Meanwhile, the result of Ag in FGS was >0.01 mg kg^{-1}. This result also proves that *W. somnifera* can accumulate silver without reducing shoot biomass, which confirms *W. somnifera*'s phytoremediation capability. Moreover, IVS accumulated 2.54% silver from suspension media treated with 200 mg kg^{-1} of $AgNO_3$ (E1D3T6) for 12 days period. With the widespread use of MNPs, an increasing amount of MNPs/metal ions are released into the natural environment daily. They entered food chains through different ecosystems (Li et al., 2023). Especially, MNPs are uptaken, translocated, and accumulated by plants, and the ratio of uptake is dependent on the type of plant species, and the ratio of accumulation also differs among plant tissues (Liu et al., 2023). NPs are great sensors, capable of detecting sensitive pollutants in different ecosystems and remediation via degradation (Ayaz Ahmed et al., 2014; Remya et al., 2022). In turn, MNPs becomes a toxic waste in the environment. Therefore, before applying them to the environment, the toxic nature of MNPs should be considered. Green synthesized metal nanoparticles (gMNPs) are easily biodegradable; they exhibit limited or no toxicity to the environment (Ying et al., 2022). Thus, the use of gMNPs must be increased and replaced in place of cMNPs.

The production of biodegradable MNPs has many advantages, which include bio-imaging, targeted drug delivery, tissue engineering, regulatable life-span of NPs and drugs in circulation (Vlasova et al., 2016) then natural degradation under biological conditions, which prevents its accumulation in living organisms and the environment. Thus, we are reporting eco-friendly and biocompatible *in planta* synthesis of Ag NPs using IVS of *W. somnifera*. This is the first step to achieving a nanopollution-free environment that prevents the accumulation of toxic cMNPs in the environment that sequentially transfers into the food chain. In the future, selecting a plant with medicinal properties and increased reduction potential, i.e., secondary metabolites, may serve as bionanofactories that can be used in nanoscience and medical sectors. The presence of metal NPs in their tissues also increases plants' therapeutics in modern medicine.

4. Conclusion

The elicitation effect of $AgNO_3$ under acute and chronic exposure periods on *in vitro* shoot cultures of *W. somnifera* is explored in this study. Among the $AgNO_3$ treated IVS highest GI (1.425 ± 0.05) and withaferin A (2.568 ± 0.08) was found in 1 mM $AgNO_3$ treatment for 12 days (E1D3T6). Further, the IVS of *W. somnifera* found to bioaccumulate 50.8 ppm Ag in their leaf tissues. The nature of the Ag in IVS was found to be Ag NPs with spherical and rod in shape and 20–40 nm in size. Thus, with proper modification, IVS of *W. somnifera* can be used as a bionanofactories for the synthesis of Ag NPs, which can be utilized for therapeutic applications without the need to go for expensive NPs isolation and purification methods. Further, in the current method, whole explants can be used for therapeutic applications. In addition, the metal reduction capability of IVS of *W. somnifera* towards other precious metal must be determined, and large-scale *in vitro* culture production using bioreactors shall increase both biomass and NPs accumulation. This study provides evidential support for research to explore the potential of hyperaccumulator/specific accumulator plant species to be utilized as bionanofactories to synthesis, store, and extract MNPs in polluted environments, thus reducing the stress on metal-polluted soils.

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Authors contributions

KS, KN, and **SN**; Conceptualization, **KN**, **KPS,** and **SV**; Methodology, investigation, and data curation, **KN** and **KA**; Software and formal analysis, **KS, SN,** and **TM**; Resources and supervision, **KN**; Original draft writing, **KA** and **KS**; Review, and editing.

Ethics approval and consent to participate

This article does not contain any studies with human or animal subjects.

Consent for publication

All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108882.

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