EGYPTIAN JOURNAL OF BOTANY (EJBO)

Establish an in vitro propagation system and produce secondary metabolites in Ashwagandha (*Withania somnifera* L.)

Amira R.M. Sallam

Department of Genetic Resources, Desert Research Center, Cairo, Egypt

Ashwagandha **(***Withania somnifera* L.) is an endangered and vital medicinal Indian plant. The present study focused on in vitro micropropagation of Ashwagandha plant material and studied the effects of Chitosan levels as an elicitor on secondary metabolic bioactivity withanolides (withanolide-A, withaferin-A). MS medium fortified by BAP (1.0mg/L) and NAA (0.125mg/L) achieved a maximum of 6 shoots per explant. In vitro shoot elongation and leaf number (12.33cm and 10.67, respectively) were improved on MS medium supplemented with BAP at 2.00mg/L and NAA at 0.25. High frequency of rooting 100% and maximum root length (8.00cm), as well as the number of white roots (1.00), were achieved on an MS medium supplemented by NAA (0.5mg/L). Referring to the impacts of Chitosan levels on *in vitro* micropropagation, all growth parameters (shoots, roots) were decreased significantly in an inverse relationship with Chitosan levels. HPLC method was used for the quantitative importance of bioactive chemical constituents, withanolide-A, and withaferin-A, to estimate the quantitative importance of bioactive chemical constituents withanolide-A, and withaferin-A affected by Chitosan levels as the elicitor *in vitro*. Chitosan at 100 ppm achieved the maximum accumulation of the withanolide-A (259.12mg/g), while the highest concentration of withaferin-A (11.96mg/g) was obtained by 200 ppm Chitosan in root tissues. In general, the results indicate the possibility of obtaining an ideal medium for the propagation of Ashwagandha *in vitro* and the importance of the effect of Chitosan as a stimulant on the vegetative parameters and increasing accumulation of active alkaloids in the shoot and root tissues of Ashwagandha.

Keywords: Chitosan, Elicitor, HPLC, Multiplication, Rooting, *Withania somnifera*, Withanolide-A, Withaferin-A.

ARTICLE HISTORY

Submitted: February 15, 2025

Accepted: Septemper 18, 2025

CORRESPONDANCE TO:

Amira R.M. Sallam

Department of Genetic Resources, Desert Research Center, Cairo, Egypt

Email: amirarasheeddrc@gmail.com

DOI: 10.21608/ejbo.2025.358579.3182

EDITED BY: Rehab Mahmoud Hafez

INTRODUCTION

Indian Ginseng or Ashwagandha *(Withania somnifera* L.) belongs to the family Solanaceae, and despite its importance as a therapeutic plant, it is endangered. Ashwagandha is commonly found in many countries like India, Pakistan, Morocco, Jordan, and Egypt. It is a perennial plant with numerous therapeutic purposes in traditional Ayurveda and modern-day medicine, for its medicinal attributes are contributed by the active constituents in its roots and leaves (Kumar, 2013; Gaurav et al., 2023). The plant possesses a broad spectrum of pharmacological properties, contains an abundance of secondary metabolites such as alkaloids, lactones, steroidal, and flavonoid, thus considered an anti-tumor, antimicrobial, anti-inflammatory, anti-stress, cardio-protective, and neuroprotective. Ashwagandha also has been used as an Anti-rheumatic, adaptogenic, anti-anxiety, anti-arthritic, liver tonic, anti-inflammatory, immunomodulatory activity, for treating sleeplessness, bronchitis, anti-depression, to cure asthma, ulcers, and dementia (Gaurav & Kumar, 2019; Gaurav et al., 2023).

The conventional method of propagating this species is by seeds. However, the viability of the seeds is very poor, and the germination percentage is low, due to a long dormancy period

that limits Ashwagandha multiplication rates. Vegetative propagation is not available for this plant. The *in vitro* technique (Tissue culture) is a valuable alternative for large-scale propagation via shoot apex and axillary bud (Jhankare et al., 2011; Khanna, 2013). For that, biotechnological advancements are urgently needed to increase yield faster. Tissue culture techniques are a practical applied biotechnological method for significant scaling healthy plants, which also makes it easier to produce high-quality planting materials, in order to preserve the genetic resources, and improve the secondary metabolites of medicinal, uncommon, and exotic plants (Ozyigit, 2023). Moreover, plant cell and tissue culture techniques used to boost the production of metabolites active biosynthesis, benzyl amino purine (BAP) and naphthalene acetic acid (NAA), Growth regulators, can induce the shoot stimulation, elongation, and regeneration. Indole acetic acid (IAA) alone and combined with indole butyric acid (IBA) can stimulate true roots *in vitro* (Ochoa-Villarreal et al., 2016; Shah, 2021; Lashin et al., 2022).

Ashwagandha consists of many chemical compounds from diverse chemical classes. These include lactones, flavonoids, and tannins in different plant parts, including roots, fruits, and aerial parts. However, traditional Indian medicine (Ayurveda) explicitly emphasizes using Ashwagandha root tissues for therapeutic purposes (Supe et al., 2011). The medicinal benefits of Ashwagandha are due to the presence of a distinct group of natural steroidal lactones called withanolides, which are mainly found in the roots (Chaurasia et al., 2000; Abdul Mazeed, 2022). Withanolides are an important secondary metabolite in *Withania somnifera* (L.) plants, which are high-value medicinal as anti-tumor and antioxidant (Ismail, 2013). A most important amongst these withanolide-A and withaferin-A, which have been shown to possess potent antioxidant activity, and have been shown to possess various therapeutic properties against diseases like anti-stress, immunomodulatory, various forms of cancer, Alzheimer's disease, Parkinson's disease, diabetes, hemopetic adaptogen, memory enhancing, anti-venom, rejuvenating, and antidiuretic properties (Sivanandhan, 2012). Withanolides-A and Withaferin-A are found in plant roots in relatively higher concentrations. On the contrary, many studies reported that a large quantity of withaferin-A and withanolides-A are not formed only in the roots but also in Ashwagandha aerial tissue (Prajapati et al., 2003). Similarly, (Pal et al., 2017; Singh et al., 2017) confirmed that different forms of withanolides are found in different plant parts. Manali (2022) and Singh et al. (2018) reported that, among withanolides, withaferin-A and withanolides-A were superior metabolites and distributed in different parts of tissues in the plant and with various concentrations.

The *in vitro* techniques can potentially initiate cells, organs, and root cultures, especially hairy root cultures, for increased withanolides production. Various studies have been conducted using in vitro techniques to explore the metabolic pathways of *W. somnifera* and the involved biosynthesis. Tissue culture techniques are used on a large scale to extract secondary metabolites from cultivated plant cells (Dhar et al., 2013; Anakha & Praveen, 2019). Tissue culture studies are also used to artificially increase the accumulation of the withanolide concentration in the plant by using various elicitors (Shah 2021; Kaur et al., 2021; Gajala, 2023). Singh (2017) explained that withanolide-A enhancement occurs in shoot tissues at levels that match native roots.

Molecules that produce secondary active metabolites and maintain their plant survival are called "Elicitors" (Namdeo, 2007; Manali, 2022). Different environmental conditions, especially biotic and abiotic stress, also affect the accumulation of withaferin-A and withanolide-A content and the productivity of *Withania somnifera*. Several abiotic and biotic elicitors, such as jasmonic acid, Chitosan, salicylic acid, and polyamines, are efficient tool that induces the plant to synthesize beneficial phytochemicals (Kim et al., 2007; ELhefny et al., 2022). Elicitors can modify plant active metabolism by encouraging the physiological stress that stimulates phytochemical bioactivity (Baenas, 2014; Narayani & Srivastava, 2017).

Elicitation is used to overcome and face the limitations of in vitro plant culture technology, which hampers its extensive commercialization. The elicitation technique is done by adding abiotic or biotic elicitors to a plant growth culture. Several factors are responsible for enhancing secondary metabolites by in vitro cultures, such as concentration, type of elicitor, growth stages, etc. (Singh et al., 2017; Singh, 2001).

Chitosan is considered a biotic elicitor that affects the in vitro culture of various medicinal plants in numerous physiological processes like photosynthesis, enhancing morphogenesis (Gupta & Jatothu, 2013; Shah, 2021). Chitosan has been effective in the pharmaceutical accumulation of important bioactive metabolites. It is a well-known

elicitor strongly affecting secondary metabolites and plant biomass production. Using it elicits the production of various secondary metabolites in most medicinal plants in vitro (Ferri & Tassoni, 2011). Under similar conditions, adding 100mg/L of Chitosan achieved higher production of withanolide compounds than aluminum chloride. Chitosan improved withanolide productivity, reaching 100mg/L in *W. somnifera* cell suspension culture (Sivanandhan et al., 2014). It also enhanced the bioactivity of withaferin-A by 69% in Ashwagandha tissues, compared with the control (Jonathan et al., 2015). In the same result, withanolide-A was (160% fold) by 100mg/L of Chitosan compared with the control. Total withaferin-A content was increased 6.3 and 5.8 times when treated with 10 ppm and 50 ppm of Chitosan, respectively. Chitosan also stimulated silymarin in milk thistle callus cultures (Gabr, 2016; Ashwaq & Alaa, 2024).

High-Performance Liquid Chromatography techniques are commonly used to analyze these extracts due to their accuracy and reliability. HPLC analysis plays a crucial role in verifying the potency and purity of Ashwagandha extracts. Specifically, it can quantify the efficacy of specific withanolides (Meena, 2021; Singh et al., 2021). HPLC methods were used to develop and assess purity to standardize the compounds. HPLC analysis provides the simplicity of isolation for the compounds, which may be markers for the standardization of the methanol extracts and preparations in *W. somnifera*. The Quantitative determinations of withanolides were quantified on the plant methanolic extracts using reverse-phase HPLC (Ismail, 2013; Singh et al., 2021).

The objectives of this work were to obtain the optimum micropropagation protocol medium for mass culture of Ashwagandha in vitro, and to study the effect of elicitor (Chitosan) on the vegetative parameters as well as the accumulation of two bioactive Alkaloids withanolides (withanolide-A, withaferin-A) in Ashwagandha tissues (shoot and root) *in vitro*, using reverse-phase HPLC for the analysis of simultaneous.

MATERIALS AND METHODS

This present study was conducted at the Tissue Culture Unit, Genetic Resources Department, Desert Research Centre, Cairo, Egypt.

Plant material

Seeds of Ashwagandha (*Withania somnifera* L.) variety Jawahar-20 were collected from Egyptian Gene Bank garden.

Culture medium

All *in vitro* experiments were conducted using **MS** (Murashige & Skoog) basal medium (Murashige & Skoog, 1962) (Sigma-Aldrich, Germany) fortified by 0.1 mg/l myoinositol, plant growth regulators (PGR) Table 1, and 30gm sucrose. The medium pH was adjusted up to 5.7 – 5.8 by using HCl 1.0 M or NaOH 1.0M. Then, after adjusting the pH, 3% (w/v) of Phytagel was added. The media was dispensed into sterilized culture tubes or jars and then sterilized at 121°C and 1.5 atmospheric pressure for 20min.

Explant preparation and sterilization

Seeds were washed many time under running tap water for 10min, and then seeds were dipped in 70% ethanol for 3 sec, and then immersion in 15% of commercial bleaching sodium hypochlorite (NaOCl 3.25%) twice for 5min. The seeds were rinsed three times in distilled and sterile water for 10min.

Seed germination

Sterilized seeds were placed under aseptic conditions in culture jars, containing sterilized cotton and 30 ml distilled water, 10- 15 seeds per jar. Seeds were cultured and incubated under 22±2°C and in full darkness conditions for 4-6 days until seeds started germination.

In vitro micropropagation

For multiplication and rooting (micropropagation) of Ashwagandha culture stock, sterilized plantlets (5: 7cm) were divided in single node and cultured in MS medium fortified with sucrose at $30gL^{-1}$, and the combination of PGRs. Ten protocols for multiplication and five protocols of rooting were recorded in Table 1, One single node cutting explants were cultured in tubes, with eight replicates for each protocol, and two tube per replicate. Cultures were incubated at 22±2C° for 16 hours photoperiod at 2000-lux, (fluorescent tubes) for 25 -30 days to induce *in vitro* proliferated shoots. For mass multiplication of stock plants, shoots were sub-cultured 3 times on the optimal multiplication medium before rooting. Survival percentages shoot number/ single node cutting, shoot length (cm), and leaves number/ shoot were recorded for the multiplication stage. Survival percentage, white-truly rooted plantlets percentage, number/ clusters, and length (cm) were estimated for the rooting stage.

Table 1. Plant growth regulators protocols used in Ashwagandha micropropagation (multiplication, rooting) in vitro

(PGRP) Protocols			
MSr Rooting treatments (MSr)	**Rooting protocols**	**MS multiplication treatments (MSm)**	**Multiplication protocols**
MS-free medium	Protocol 1	MS-free medium	Protocol 1
0.125mg/L NAA	Protocol 2	0.50mg/L BAP + 0.00mg/L NAA	Protocol 2
0.25mg/L NAA	Protocol 3	1.00mg/L BAP + 0 00mg/L/ l NAA	Protocol 3
0.50mg/L NAA	Protocol 4	2.00mg/L BAP + 0.00mg/L NAA	Protocol 4
1.00mg/L NAA	Protocol 5	0.50mg/L BAP + 0.125mg/L NAA	Protocol 5
--	--	1.00mg/L BAP + 0. 125mg/L NAA	Protocol 6
--	--	2.00mg/L BAP + 0.125mg/L NAA	Protocol 7
--	--	0.50mg/L BAP + 0.25mg/L NAA	Protocol 8
--	--	1.00mg/L BAP + 0. 25mg/L NAA	Protocol 9
--	--	2.00mg/L BAP + 0.25mg/L NAA	Protocol 10

- MSm; MS multiplication medium protocol.
- MSr; MS rooting medium protocol.

Effect of Chitosan levels *in vitro*

The effect of the elicitor Chitosan on vegetative parameters and the accumulation of two bioactive alkaloids withanolides (withanolide-A & withaferin-A) in Ashwagandha shoot and root tissues *in vitro* was studied.

1. *In vitro* micropropagation with Chitosan

In vitro propagated plantlets were divided into single-node cuttings and cultured in tubes containing 10ml of the optimum multiplication MS medium fortified with different Chitosan levels (0.00, 25, 50, 100, 200 ppm) as presented in Table 2. Twelve tubes with two explants in each tube were considered as a treatment. Cultures were incubated at 22±2°C under a 16h photo period (2000-lux, daily fluorescent tubes). Survival% shoot numbers, shoot length (cm)/ single node cutting, leave numbers/ shoot, root numbers, and length (cm) were counted for cluster after four weeks of growth.

2. Determination of withanolide-A and withaferin-A by HPLC method

The quantitative analysis of withanolide-A & withaferin-A, carried out *in vitro* for Ashwagandha shoot and root tissues under the effect of Chitosan levels, using the reversed-phase HPLC method, according to Ganzera et al. (2003). Withanolide-A and withaferin-A Standards were purchased by Organic Nation Egypt Ashwagandha extract (withanolide-A and withaferin-A 5%). The standard was prepared from (1mg/mL) of withanolide-A and withaferin-A, using HPLC grade methanol, and then kept at −20 °C until used. Working solutions were prepared from 70% (v/v) methanol. Samples and standards were filtered over 0.45 µm. The separation compounds were performed on a (Ultimate 3000) C18, thermal column (P.S. 250mm * I.D. 4.6mm 5µm). Mobile phase consisted of acetonitrile: water (45:55 V/V) in an isocratic elution at a 0.8 ml/min flow rate. The column temperature is maintained at 27°C. Analyses detection was performed by using an SPD-M20A detector with a photodiode array.

The chromatography system was equilibrated with the mobile phase using the HPLC technique. A total of 10µl of samples were injected into the injection port of the HPLC equipment using a Hamilton syringe. Samples were allowed to run for the preset runtime. The time of retention and peak area of the peak were observed. A standard curve helped quantify withanolides' bioactivity in the samples (Singh et al., 2018). Metabolite contents were expressed in mg/DW (mg/dry weight).

Table 2. Chitosan levels used in the in vitro experiment

MS plus Chitosan levels	Treatments
MS-free formula	CH 0
25mg/L Chitosan	CH 1
50mg/L Chitosan	CH 2
100mg/L Chitosan	CH 3
200mg/L Chitosan	CH 4

- CH; MS multiplication medium with Chitosan levels.

The Statistics of analysis and experimental designs

A completely randomized design form was used for all Experiments. Recorded data were statistically analyzed using the analysis of variance (ANOVA) technique. Statistical analysis was performed using standard procedures for a completely randomized design by analysis of variance. Separation of means was performed to compare the different b between the means, range test described by Duncan (1955) by different letters at a significance level of P≤ 0.05.

RESULTS AND DISCUSSION

The present study was conducted to obtain the optimum micropropagation medium for mass production of Ashwagandha *in vitro* and to study the impact of Chitosan levels on vegetative parameters as well as the accumulation of bioactive alkaloids (withanolide-A and withaferin-A) in the plant.

In vitro multiplication

Mass multiplication production of Ashwagandha was induced in vitro Figure 1, sub-culturing the healthy single-node cuttings three times. MS medium protocols (1- 10) were fortified with different combinations (Table 1) (0.00, 0.50, 1.00, 2.00mg/L) of BAP and (0.00, 0.125, 0.25mg/L) of NAA. Rani et al. (2014) reported that a combination of NAA and BAP was more effective than BAP alone for shoot production and multiplication. In Ashwagandha, BAP played an important key role (Panwar et al., 2011; Ojha & Choudhary, 2013). Rani et al. (2014) observed that different combinations of NAA and BAP showed the best results for shoot elongation and regeneration in Ashwagandha. Data in Table 3 and Figure (2-4, 5) illustrate that all treatments' survival **%** was 100%. Shoot numbers ranged between 1 and 6 shoots per single node cutting, and 6 shoots was the highest mean number of shoots recorded on protocol 6 MS medium with 1.00mg/L BAP and 0.125mg/L NAA. This result matches Sivanesan & Murugesan (2008), who found that the maximum of 17 shoots were induced from the Ashwagandha nodal explants in BAP at 1.0mg/L. Manickam et al. (2000) also reported that the highest number (8) of Ashwagandha shoots was obtained by nodal explants cultured on protocol 3 MS medium with 1mg/L BAP. Irshad et al. (2013) also reported that axillary and apical buds of Ashwagandha gave maximum response on MS medium with 1mg/L BAP. Results in Table 3 also showed that BAP-containing medium at 2.00mg/L with 0.25 NAA (protocol 10) gave the maximum mean values of shoot length (12.33cm) and leaves number (10.67). Similar results were also observed by Rishikesh et al. (2016), who using MS media with BAP (2.0mg/L) produced the maximum average shoot length in Ashwagandha. In the same context also Tamara et al. (2011) reported that the highest vegetative parameters (number of shoots, shoot length, average number of leaves/ explants) were achieved on MS medium with BA at 2.0mg/L and NAA at 0.1mg/L in the important medicinal plant (*Rollinia mucosa*, and *Teucrium polium* L) respectively.

Figure 1. Ashwagandha mass multiplication steps induced in vitro

Table 3. The effect of plant growth regulators (PGR) protocols on *in vitro* multiplication parameters/ clusters of Ashwagandha after 30 days

MSm Protocols	Parameters			
	Survival %	Shoot Number/ cluster	Shoot Length (cm)	Leaves Number/ cluster
Protocol 1	100 a	1.33 c	5.00 d	4.00 e
Protocol 2	100 a	2.00 bc	6.67 cd	5.00 de
Protocol 3	100 a	2.00 bc	7.00 cd	7.00 bcd
Protocol 4	100 a	3.00 bc	7.00 cd	7.00 bcd
Protocol 5	100 a	3.00 bc	6.67 cd	6.66 bcde
Protocol 6	100 a	6.00 a	8.00 bcd	6.33 cde
Protocol 7	100 a	4.33 ab	9.00 abc	7.00 bcd
Protocol 8	100 a	2.00 bc	7.00 cd	8.00 abc
Protocol 9	100 a	5.67 a	11.00 ab	9.33 ab
Protocol 10	100 a	4.33 ab	12.33 a	10.67 a

- MSm; MS multiplication medium protocol.

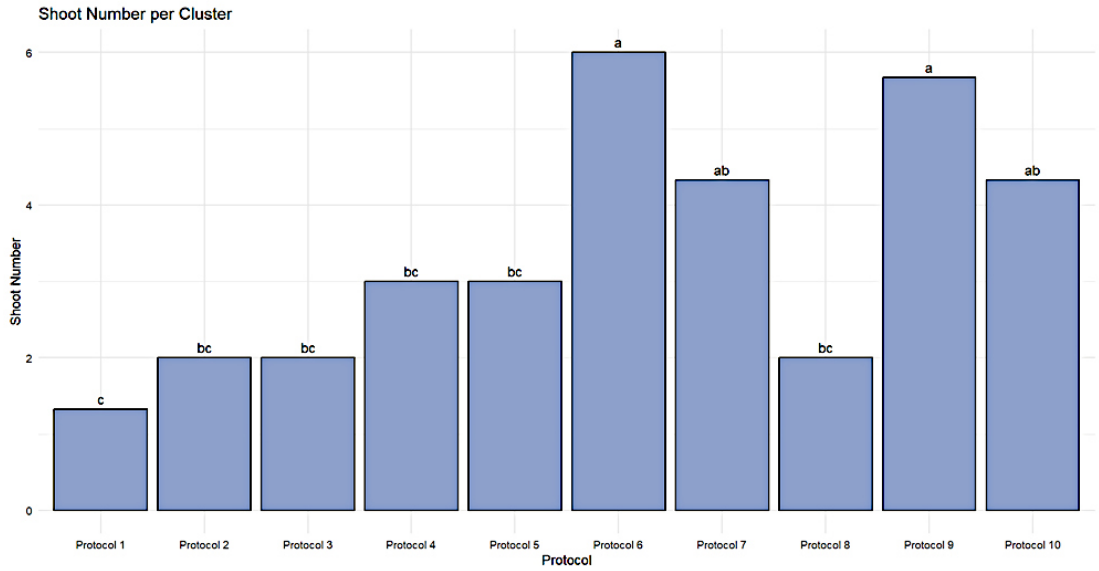

Figure 2. Shoot numbers recorded in vitro per single node cutting, for 10 protocols (Table 3)

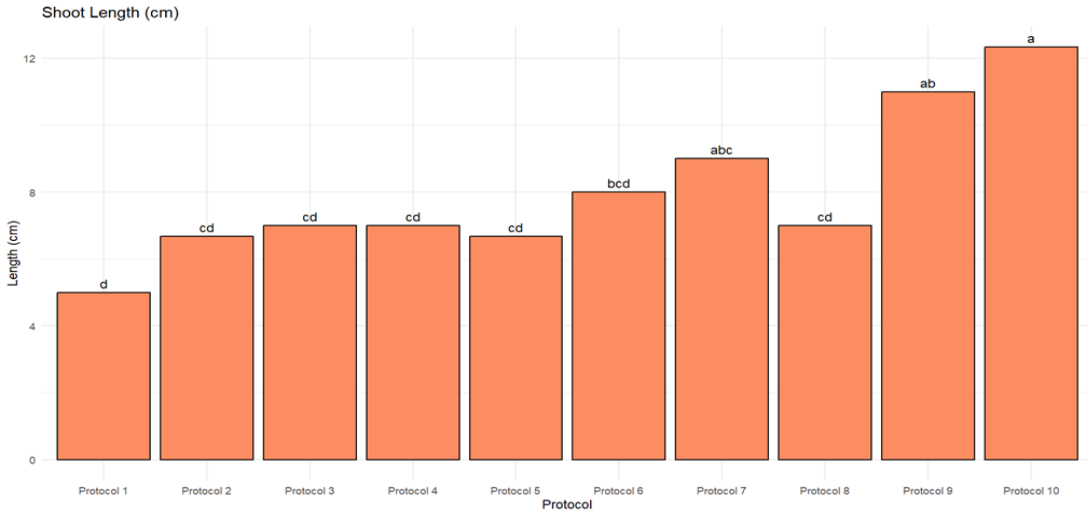

Figure 3. Shoot length (cm) recorded in vitro per single node cutting, for 10 protocols (Table 3)

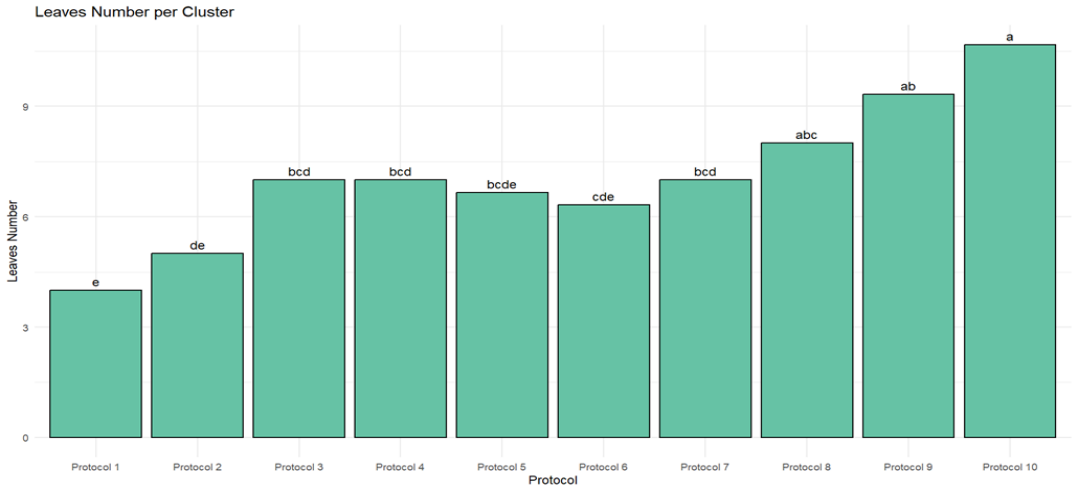

Figure 4. Leaves numbers recorded in vitro per single node cutting, for 10 protocols (Table 3).

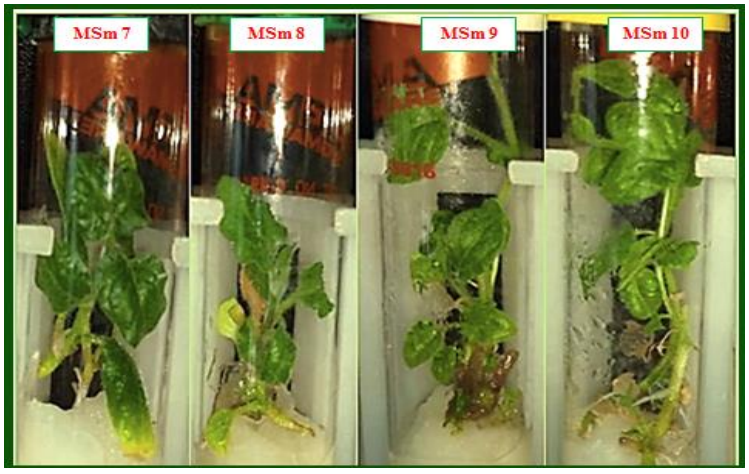

Figure 5. In vitro culture of shoots on MS medium protocols (MSm 7-10) of Ashwagandha explants

In vitro rooting

MS rooting medium was supplemented with different NAA concentrations (five protocols), Table 1, for the induction of transparent and white-true rooted plantlets *in vitro*. In general, transparent roots were first formed, and then plantlets formed white-roots. Table 4 and Figure 6 demonstrate the impact of different NAA concentrations on the rooting parameters (white-true roots) in Ashwagandha *in vitro*. Tamara et al. (2011) reported that NAA was the only growth regulator that promotes root induction in the *Teucrium polium* L. medicinal plant. The survival percentage reached 100% on all treatments except for the highest NAA concentration, which gave 91.67 % without any significant difference between the treatments. The rooted explants percentage reached 100% with one (1.00) root number/plantlet recorded on both NAA at 0.50mg/L and 1.00mg/L (protocols 4 and 5). Krishnamurthy & Patel (2013) observed that *W. somnifera* regenerated shoots produced a maximum (80-90%) rooting percentage with 4-5 roots/ shoot when cultured on MS media with 0.4mg/L NAA. Also, the highest mean value of whit root length (8.00cm) was recorded at 0.50mg/L of NAA (protocol 4). Control MS medium and NAA at 0.125mg/L (protocols 1 and 2) did not record significant values. The same results were investigated by Sivanesan & Murugesan, (2008), who referred that addition of NAA, showed that the highest rooting response (%), as well the highest roots number of Ashwagandha observed in MS medium with NAA (1mg/L), while the highest mane value of roots length was recorded on NAA at 0.1mg/L. Also, Tamara et al. (2011), found that adding NAA only promotes the best rooting parameters (number and length of roots) in the medical plant.

Table 4. The effect of NAA protocol concentrations on the white-true roots induction after 40 days in Ashwagandha in vitro

MSr Protocols	Parameters			
	Survival%	White true rooted % plantlets/ cluster	White, true root/ plantlet	White, true root length. (cm)
Protocol 1	100.00 a	0.00 c	0.00 b	0.00 c
Protocol 2	100.00 a	0.00 c	0.00 b	0.00 c
Protocol 3	100.00 a	61.6 b	0.67 ab	2.33 bc
Protocol 4	100.00 a	100.00 a	1.00 a	8.00 a
Protocol 5	91.67 a	100.00 a	1.00 a	4.00 b

- MSr; MS rooting medium protocol.

Figure 6. In vitro white-true roots induction on MS media protocols (MSr 1-5) of Ashwagandha plantlets

In vitro effect of Chitosan

Chitosan is a polysaccharide compound more soluble than chitin. It is used as a biotic elicitor produced from shrimp shells. The impact of Chitosan levels (Table 2) as an abiotic elicitor on the vegetative parameters and the contents of two types of withanolides (withanolides-A and withaferin-A) in Ashwagandha tissues (shoots and roots) *in vitro* was studied.

Effect of different Chitosan levels on *in vitro* vegetative growth

Compared to the control media, the results show that all growth parameters decreased significantly as Chitosan levels increased. Most biotic and abiotic elicitors have an inhibitory impact on the vegetative parameters of the plant. The degree of the effect often varies according to the type of elicitor, its level, and exposure time. According to Shah (2021), vegetative biomass production depends on Chitosan concentration; higher Chitosan concentration up to 5.0 ppm decreases the biomass production in *Silybum marianum* (L.). Regarding survival percentage, the data in Table 5 and Figure 7 demonstrate that it reached 100% by 25.00 ppm and 50 ppm of Chitosan and MS control medium. The highest mean values (4.00, 9.00cm, and 7.00, respectively) were recorded on MS control medium (protocol 1) for shoot number, length, and number of leaves. In contrast, the significantly lowest mean values were recorded at the highest level of Chitosan (200 ppm). The highest average true roots number and length (1 and 8cm, respectively) were obtained on the control treatment, and the lowest mean values (0.33 and 1.17cm) were obtained using 200 ppm. In the same way, Ashwaq & Alaa (2024) observed that adding Chitosan significantly reduced Ashwagandha callus parameters (more than 70% decreases) compared with the control MS medium.

Table 5. The effect of different Chitosan levels on the Ashwagandha vegetative parameters in vitro

Chitosan levels treatments (ppm)	Parameters					
	Survival %	Shoots number/ cluster	Shoot length (cm)	Leaf number/ cluster	True-roots Number/ plantlets	True-roots length (cm)
CH 0	100.00 a	4.00 a	9.00 a	7.00 a	1.00 a	8.00 a
CH 1	100.00 a	2.67 ab	6.33 ab	6.33 ab	1.00 a	2.16 b
CH 2	100.00 a	1.77 bc	4.67 bc	5.00 ab	1.00 a	2.67 b
CH 3	85.67 a	1.7 bc	4.26 bc	3.76 bc	1.00 a	3.83 b
CH 4	16.67 b	0.33 c	1.50 c	1.00 c	0.33 b	1.17 d

- CH; MS multiplication medium with Chitosan levels.

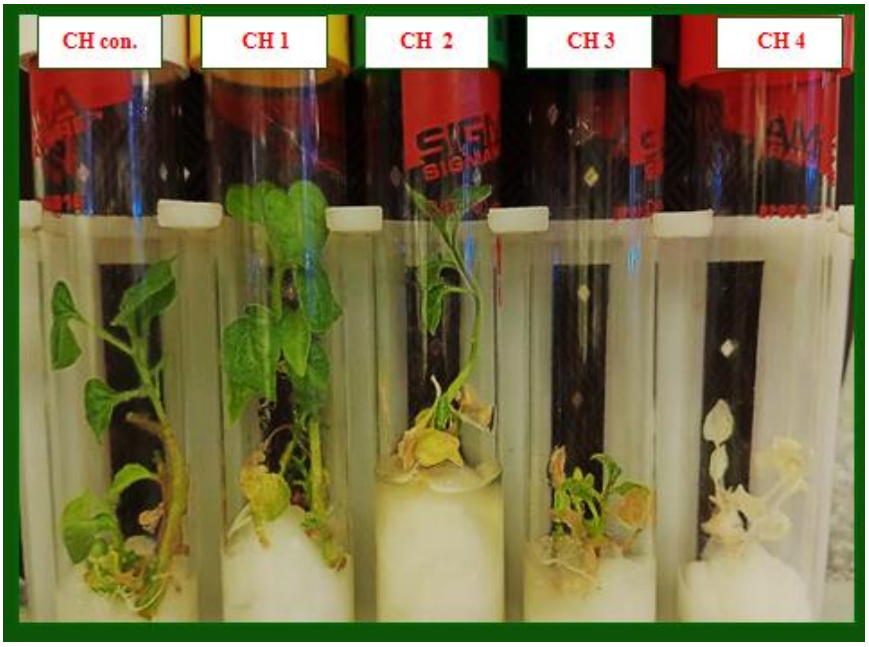

Figure 7. In vitro effect of different Chitosan levels on the vegetative parameters of Ashwagandha plantlets

Effect of different Chitosan levels on the withanolide-A and withaferin-A content.

Withanolide alkaloids are naturally found accumulating in most species of the *Withania* genus, especially *Withania somnifera* (Ashwagandha). Researchers found that withanolide-A and withaferin-A are distributed in the Ashwagandha plant parts (Baenas et al., 2014). Elicitors are stress, enhance secondary metabolites synthesis, or produce natural plant products from abiotic and biotic sources. To estimate the impact of Chitosan levels on stimulating and the accumulation of withanolides, an HPLC method for profiling withanolide-A, withaferin-A as mg/ g of dry tissues in both in vitro shoots and roots of Ashwagandha was used. Although the inhibition impacts of elicitors on the vegetative growth of plants, it also increases the activity of secondary metabolism in these cultures, producing secondary metabolites. The data obtained in this investigation showed that the increase in Chitosan levels directly increased concentrations of both withanolide-A and withaferin-A in plant tissues compared to the MS control media. Also, Santosh (2012) noticed a significant accumulation enhancement on the yield of withanolides-A and withaferin-A observed in the controlled environment relative to the wild environment. The current study on Ashwagandha, considering withanolide-A, found that it was highly accumulated in root tissue compared to the shoot tissue. Dhar (2013) referred that Chitosan caused an increase in the accumulation of withanolides in the Ashwagandha adventitious root in vitro. Data in Table 6 and Figures 8-17 show that highest concentration

of withanolide-A (259.12mg/g dry Wight) was obtained using 100 ppm Chitosan in root tissues, followed by 193.32 mg/g dry Wight which recorded by 200 ppm Chitosan with shoot tissues, as well the lowest concentration (53.94mg/g) in shoot tissues, followed by 74.54 mg/g with root tissue were obtained on MS control medium without Chitosan. The same results were obtained by Ashwaq & Alaa (2024, who reported that Chitosan at 100 ppm stimulates the biosynthesis of 27-OH-withanolide-A with a 160% increase compared to the control, in Ashwagandha callus tissues, for Withaferin-A accumulation correlated positively with Chitosan levels. The highest concentrations (11.96 and 11.84mg/g dry Weight) in root tissue were recorded respectively at 200 and 100 ppm Chitosan. Meanwhile, the lowest value of 0.88mg/g was obtained in root tissue, followed by 1.59 mg/g in shoot tissues on MS control medium. No recorded values of withaferin-A were noticed for Chitosan levels (50 and 100 ppm) in shoot tissue and (25 and 50 ppm) in root tissues. Sivanandhan et al. (2012) proved that the Chitosan levels at 100 mg/l stimulated the accumulation of withanolides in the adventitious cultures of Ashwagandha root, compared to aluminum chloride treatment. As for Singh (2022), Chitosan application increased the accumulation of withanolides-A by 11.3-fold *in vitro*. Also, the maximum withanolide-A and withaferin-A content was recorded by Chitosan (100 ppm) in leaves, stems, and root tissues of Ashwagandha. Root thickness was increased directly with increasing Chitosan levels

Table 6. Effect of different Treatments, Chitosan levels (25, 50, 100, 200 ppm), on the quantification of total withanolides (withanolide-A and withaferin-A) content in Ashwagandha shoots and roots tissue after 45- 60 days in vitro

MS+ Chitosan levels ppm	Quantification of withanolides (mg/ g dry weight)			
	Shoots		Roots	
	Withanolide-A	Withaferin- A	Withanolides -A	Withaferin - A
CH 0	53.94	1.59	74.54	**0.88**
CH1	81.23	1.82	77.88	--
CH 2	92.36	--	154.88	--
CH 3	185.17	--	259.12	**11.84**
CH 4	193.32	2.44	161.39	**11.96**

- CH; MS multiplication medium with Chitosan levels.

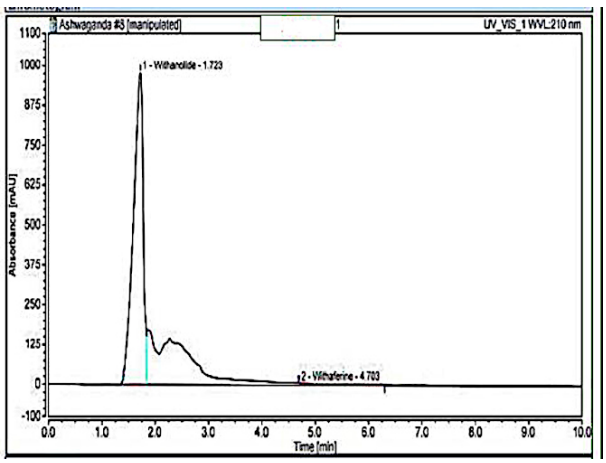

Figure 8. HPLC chromatogram of standards withanolide-A & withaferin-A in shoot tissue samples on MS control medium

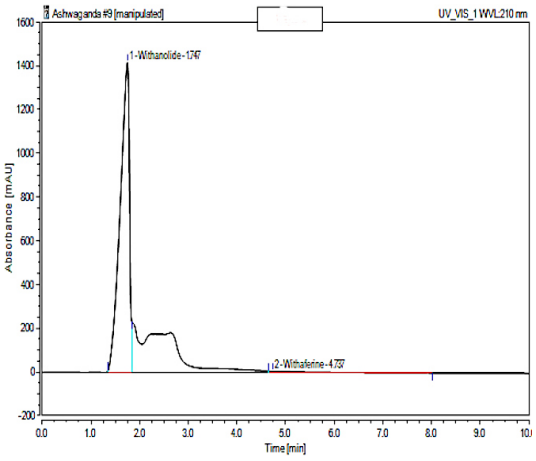

Figure 9. HPLC chromatogram of standards withanolide-A & withaferin-A in shoot tissue samples on MS + (25 ppm) Chitosan medium

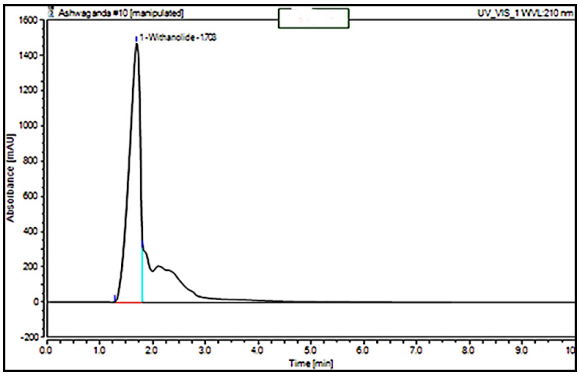

Figure 10. HPLC chromatogram of standards withanolide-A & withaferin-A in shoot tissue samples on MS + (50 ppm) Chitosan medium

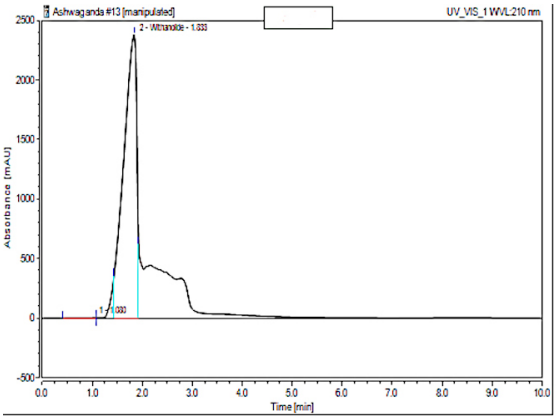

Figure 11. HPLC chromatogram of standards withanolide-A and withaferin-A in a shoot tissue sample on MS + (100 ppm) Chitosan medium

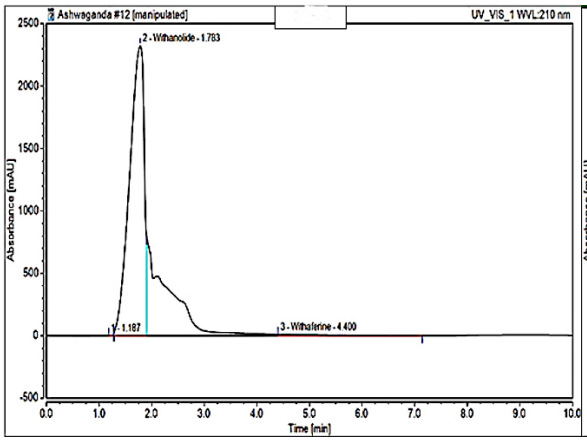

Figure 12. HPLC chromatogram of standards withanolide-A & withaferin-A in shoot tissue samples on MS + (200 ppm) Chitosan medium

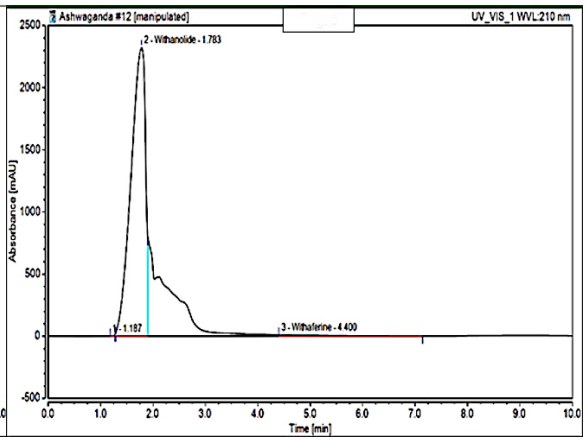

Figure 13. HPLC chromatogram of standards withanolide-A & withaferin-A in root tissue samples on MS control medium

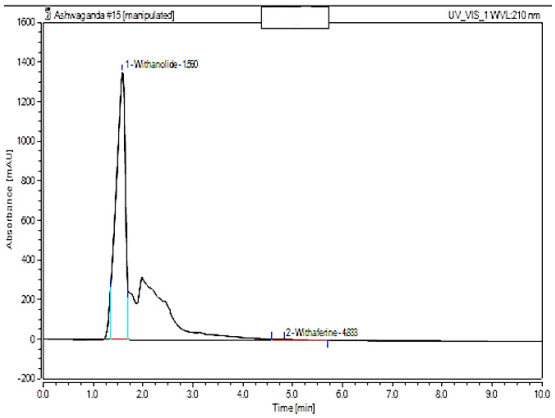

Figure 14. HPLC chromatogram of standards withanolide-A & withaferin-A in root tissues sample on MS + (25ppm) Chitosan control medium

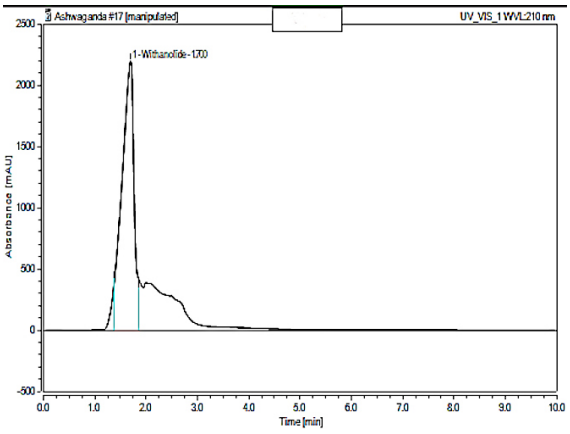

Figure 15. HPLC chromatogram of standards withanolide-A & withaferin-A in root tissues sample on MS + (50ppm) Chitosan control medium

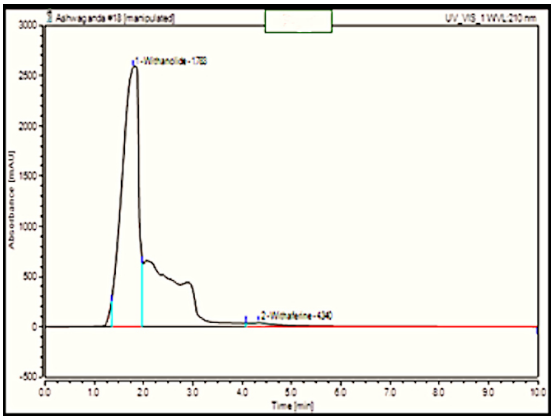

Figure 16. HPLC chromatogram of standards withanolide-A & withaferin-A in root tissues sample on MS + (100ppm) Chitosan control medium

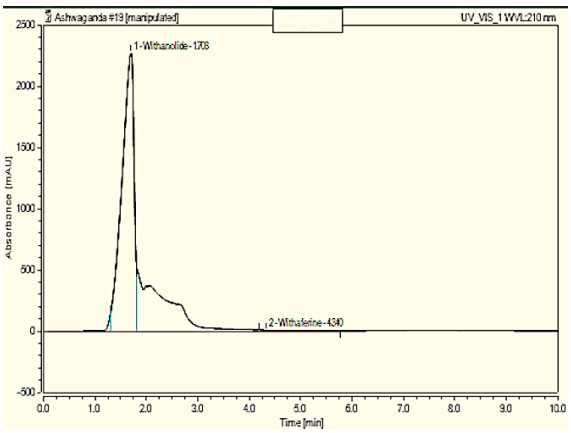

Figure 17. HPLC chromatogram of standards withanolide-A & withaferin-A in root tissues sample on MS + (200ppm) Chitosan control medium

CONCLUSION

Ashwagandha has proven to be important in pharmaceutical industries because it is rich in the varied bioactive compounds (withanolides), which are most available in various plant parts. Also found that the elicitors, such as Chitosan, were very effective in inducing the enhanced accumulation of withanolide-A and withaferin-A in the plant tissues in vitro. Three experiments were conducted in this investigation. For *in vitro* propagation, MS media fortified by BAP alone or with NAA achieved the maximum micropropagation parameters. White-true roots were produced only by MS with NAA protocols. Chitosan at high levels achieved the maximum withanolide-A and withaferin-A in the root tissues. Also, adding Chitosan to culture media decreased all micropropagation parameters, which negatively correlated with increased Chitosan levels. HPLC was used to estimate the quantitative content of withanolides affected by Chitosan levels. In future investigations, we will need more attention to establishing a true roots culture system in Ashwagandha as a promising application for biomass accumulation of secondary metabolic bioactivity (withanolides) production, to present a broad spectrum of pharmacological, and to overcome many health problems. Also, studies have shown that withanolides can be obtained from plant shoots in quantities approaching those existing in the roots. This method can be more sustainable because it does not require uprooting the entire plant, allowing regrowth and multiple harvests. Ashwagandha is a drought-tolerant plant that can grow especially in arid and semiarid regions and help conserve water resources. Also, finally, conservation of Ashwagandha germplasm is an essential demand to ensure future availability and diversity for sustainability.

Acknowledgment: I am eternally grateful to my dear Dr. Ghada A. Hegazi, Professor of Plant Biotechnology, Dr. Aymen E. Badran, Professor of Plant Breeding, and Dr. Gamil R. Aboueldis, Researcher of Plant Biotechnology, who supported me in this work.

Ethical approval: Not applicable.

REFERENCES

Abdul Mazeed, Pooja M., Dipender K., Priyanka S. (2022). The enhancement of root yield and quality of Ashwagandha (*Withania somnifera* L.) Dunal, by weeds leaves extracts. *Indian Journal of Weed Science*, 54(1): 81–86.

Anakha, R.N., Praveen, N. (2019). Biochemical and phytochemical variations during the growth phase of *Withania somnifera* (L.) Dunal. *Journal of Pharmacognosy and Phytochemistry,* 8(3): 1930-1937.

Ashwaq S.A., Alaa J.T. (2024). Biotic and abiotic elicitors induce biosynthesis and accumulation of some Withanolide compounds in the callus culture of *Withania somnifera* L. *Egyptian Journal of Botany,* 64(1): 245-256.

Baenas, N., García-Viguera, C., Moreno, D.A. (2014). Elicitation: A tool for enriching the bioactive composition of foods. *Molecules*, 19: 13541–13563.

Chaurasia, S.S., Panda, S., Kar, A. (2000). *Withania somnifera* root extract in the regulation of lead-induced oxidative damage in male mouse.

Pharmaceutical Research, 41(6): 663–666.

Dhar, N., Rana, S., Bhat, W.W., Razdan, S., Pandith, S.A., Khan, S., et al. (2013). Dynamics of Withanolides biosynthesis about temporal expression pattern of metabolic genes in *Withania somnifera* (L.) Dunal: A comparative study in two morpho-chemovariants. *Molecular Biology Reports*, 40: 7007–7016.

Duncan, D.B. (1955). Multiple range and Multiple F. test. *Biometrics*, 11(1): 1-42.

ELhefny, Mervat, Mamoun, S.M., Abd elkareem, M., Salem, Z.M. (2022). GC-MS and HPLC analyses of phytochemical compounds from *Withania somnifera* L. leaves extract. *Alexandria Journal of Agricultural Sciences*, 67(1): 11-17.

Ferri , M., Tassoni A. (2011). Chitosan as elicitor of health beneficial secondary metabolites in in vitro plant cell cultures. In: "*Handbook of Chitosan Research and Applications*", Mackay R.G., Tait J.M., (Eds.), pp 389–413. Nova Science Publishers, New York.

Gabr, A.M.M., Mabrok, H.B., Ghanem, K.Z., Blaut, M., Smetanska, I. (2016). Lignan accumulation in callus and Agrobacterium rhizogenes-mediated hairy root cultures of flax (*Linum usitatissimum*). *Plant Cell, Tissue and Organ Culture,* 126: 255–267.

Gajala, A., Alice T., Vivek K.S. (2023). The past, present and future aspects of *Withania somnifera* (L.) Dunal breeding: A review. *The Pharma Innovation Journal,* 12(7): 1928-1935.

Ganzera, M., Choudhary, M.I., Khan, I.A. (2003). Quantitative HPLC analysis of withanolides in *Withania somnifera. Fitoterapia,* 74(1-2): 68-76.

Gaurav, N.M., Kumar A. (2019). Effect of growth regulators on in vitro callusing of wild variety of *Withania somnifera* L. in B5 medium. *Indian Forester,* 145(12): 1176-81.

Gaurav, N., Ruchi S., Ashwani P., Kumari S., Akansha R., Natasha C., et al. (2023). Effect of growth regulators on direct in vitro culture and clonal propagation of *Withania somnifera*. *European Chemical Bulletin,* 12(Special Issue 5): 5112 – 5118.

Gupta, S.D., Jatothu, B. (2013). Fundamentals and applications of light-emitting diodes (LEDs) in in vitro plant growth and morphogenesis. *Plant Biotechnology Reports,* 7: 211–220.

Irshad, B.A., Alia, A., Saxena, R.C., Itoo, A., Kumar, S., Ahmed, M. (2013). In vitro propagation of *Withania somnifera* (L.) Dunal (Ashwagandha) an endangered medicinal plant. *International Journal of Pharmaceutical Science Invention,* 3(6): 349-355.

Ismail, S.K. (2013). Quantitive evolution of Withanolides content of Egyptian *Withania somnifera* (L.) Roots. *Al-Azhar Journal of Pharmaceutical Sciences,* 47: 95-104.

Jhankare, A., Tiwari, G., Tripathi, M.K., Baghel B.S., Tiwari, S. (2011). Plant regeneration from mature cotyledon, embryo and hypocotyls explants of *Withania somnifera* (L.) Dunal. *Journal of Agricultural Technology*, 7(4): 1023-1035.

Jonathan, G., Rivka, R., Avinoam, S., Lumír, H., Nirit, B. (2015). Hypoglycemic activity of withanolides and elicitated *Withania somnifera*. *Phytochemistry*, 116(1): 283–289.

Kaur, K., Singh, P., Kaur, K., Bhandawat, A., Nogia, P., Pati, K.P. (2021). Development of robust in vitro culture protocol for the propagation of genetically and phytochemically stable plants of *Withania somnifera* (L.) Dunal (Ashwagandha). *Industrial Crops & Products,* 166: 1-15.

Khanna, Punit K., Kumar, A., Chandra, R., Verma, V. (2013). Germination behaviour of seeds of *Withania somnifera* (L.) Dunal: A high value medicinal plant. *Physiology and Molecular Biology of Plants*, 19(3): 449–454

Kim, O.T., Bang, K.H., Shin, Y.S., Lee, M.J., Jung, S.J., Hyun, D.Y., et al. (2007). Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. *Plant Cell Reports,* 26(11): 1941–1949.

Krishnamurthy, R., Patel, P. (2013). Feasibility studies on in vitro mass-scale propagation of Indian Ashwagandha (*Withania somnifera*) cultivars for commercial purpose. *Journal of Pharmacognosy and Phytochemistry,* 2(2): 168-174.

Kumar, R., Varma, D., Namboothiri, I.N.N. (2013). Synthesis of withasomnines and their non-natural analogues from aldehydes and 4-Nitro-1-butanol in three steps. *Journal of Organic Chemistry,* 78: 3482-3486.

Lashin, I., Aref, M., Abd El-Kader, E., Heussin, E., Ewais, E. (2022). Antimicrobial and in vitro *Dillenia indica* L. *Egyptian Journal of Botany*, 62(1): 213-226.

Manali, S., Sanjeev, A., Obaid, A., Abdulmalik, S.A. Altamimi, A.R., Nawaf, A., et al. (2022). Optimization of elicitation conditions to enhance

the production of potent metabolite Withanolide from *Withania somnifera* (L.). *Metabolites*, 12(9): 854.

Manickam, V.S., Mathavan, R.E., Antonisamy, R. (2000). Regeneration of Indian ginseng plantlets from stem callus. *Plant Cell, Tissue and Organ Culture,* 62: 181-185.

Meena, A.K. (2021). Estimation of Withaferin-A By HPLC and standardization of the Ashwagandha Lehyam formulation. *Heliyon*, 7(2): e06116.

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

Namdeo, A.G. (2007). Plant cell elicitation for production of secondary metabolites: A review. *Pharmacognosy Reviews,* 1(1): 69–79.

Narayani, M., Srivastava, S. (2017). Elicitation: A stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochemistry Reviews,* 16: 1227–1252.

Ochoa-Villarreal, M., Susan, H., Hong, S.M., Jang, M.O., Jin, Y.W., et al. (2016). Plant cell culture strategies for the production of natural products. *BMB Reports*, 49(3):149–158.

Ojha, A., Choudhary, R. (2013). Callus development and indirect shoot regeneration from leaves and stem explants of *Stevia rebaudiana* bertoni. *Journal of Cell and Tissue Research*, 13(2): 3755-3759.

Ozyigit, I.I., Dogan I., Hocaoglu-Ozyigit, A., Yalcinm, B., Erdogan, A., Yalcin, I.E., et al. (2023). Production of secondary metabolites using tissue culture-based biotechnological applications. *Frontiers in Plant Science,* 14: 1132555. 10.3389/fpls.2023.1132555

Pal, S., Yadav, A.K., Singh, A.K., Rastogi, S., Gupta, M.M., et al. (2017) Nitrogen treatment enhances sterols and withaferin-A through transcriptional activation of jasmonate pathway, WRKY transcription factors, and biosynthesis genes in *Withania somnifera* (L.) Dunal. *Protoplasma*, 254(1): 389–399.

Panwar, G.S., Attitalla, I.H., Guru, S.K. (2011). An efficient in vitro clonal propagation and estimation of reserpine content in different plant parts of *Rauwolfia serpentina*. *American-Eurasian Journal of Scientific Research*, 6 (4): 217-222.

Prajapati, N.D., Purohit, S.S., Shanna A.K., Kumar A. (2003). "*A Hand Book of Medicinal and Aromatic Plants A Complete Source Book, Section II Medicinal Plants A to Z (2003)*". Published by Agro bios India Agro House, Jodhpur. 312p.

Rani, A., Kumar M., Sanjeev K. (2014). In vitro propagation of *Withania somnifera* (L.) Dunal. from shoot apex explants. *Journal of Applied and Natural Science,* 6(1): 159-163 JANS.

Rishikesh, H. Autade, S., Fargade, A., Amol R., Savant, S., Gangurde, S., et al. (2016). Micropropagation of Ashwagandha (*Withania somnifera*). *Bioscience Biotechnology Research Commmunications*, 9(1): 88-93 (2016).

Santosh, T.D., Yogesh, S.B., Suresh, D.J., Mahabaleshwar, V.H. (2012). Quantification of major bioactive Withanolides in *Withania somnifera* (Ashwagandha) roots by HPTLC for rapid validation of Ayurvedic products. *Journal of Planar Chromatography*. 25(4): 290-294.

Shah, M., Jan, H., Drouet, S., Tungmunnithum, D., Shirazi, J.H., Hano, C., et al. (2021) Chitosan elicitation impacts flavonolignan biosynthesis in *Silybum marianum* (L.) gaertn cell suspension and enhances antioxidant and anti-Inflammatory activities of cell extracts. *Molecules,* 26(4): 791.

Singh, B., Saxena, A.K., Chandan, B.K., Gupta, D.K., Bhutani, K.K., Anand, K.K. (2001) Adaptogenic activity of a novel, withanolide-free aqueous fraction from the roots of *Withania somnifera* Dun. *Phytotherapy Research,* 15: 311–318.

Singh, M., Shah, P., Punetha, H., Gaur, A.K., Kumar, A., Agrawal, S. (2017). Isolation and quantification of a potent anti-cancerous compound, Withaferin A from the aerial parts of *Withania somnifera* (Ashwagandha). *Anaerobic Digestion Plant Sciences,* 30(II): 231–235.

Singh, M., Shah, P., Punetha, H., Agrawal, S.b. (2018). Varietal comparison of Withanolide contents in different tissues of *Withania somnifera* (L.) Dunal (Ashwagandha). *International Journal of Life-Sciences Scientific Research,* 4: 1752–1758.

Singh, M., Agrawal, S., Afzal, O.. Altamimi, A.S.A., Redhwan, A., Alshammari, N., et al. (2022). Optimization of elicitation conditions to enhance the production of potent metabolite Withanolide from *Withania somnifera* (L.). *Metabolites,* 12(9): 854.

Singh, N., Yadav, S.S., Rao, A.S., Nandal, A., Kumar, S., Ganaie S.A., et al. (2021). Review on anticancerous therapeutic potential of *Withania somnifera* (L.) Dunal. *Journal of Ethnopharmacology*, 270:

113704. doi: 10.1016/j.jep.2020.113704.

Sivanandhan, G., Arun, M., Mayavan, S. (2012). Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of Withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. *Applied Biochemistry and Biotechnology,* 168(3): 681-696.

Sivanandhan, G., Selvaraj, N., Ganapathi, A., Manickavasagam, M. (2014). Enhanced biosynthesis of withanolides by elicitation and precursor feeding in cell suspension culture of *Withania somnifera* (L.) Dunal in shake-flask culture and bioreactor. *PLoS ONE* , 9(8):1–11.

Sivanesan, I., Murugesan, K. (2008). An Efficient regeneration from nodal explants of *Withania somnifera* Dunal. *Asian Journal of Plant Sciences,* 7: 551-556.

Supe, U., Dhote, F., Roymon, M.G. (2011). A review on micro propagation of *Withania somnifera* – A medicinal plant. *Journal of Agricultural Technology,* 7(6): 1475-1483

Tamara, S., Al-Qudah, Rida, A., Shibli, F., Alali, Q. (2011), *In vitro* propagation and secondary metabolites production in wild germander (*Teucrium polium* L.). *In Vitro Cellular & Developmental Biology – Plant*, 47: 496-505.