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Research Article

Impact of media type on callus morphology and standardization of *Withania somnifera* cell culture for enhanced secondary metabolite production

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Abstract

The medicinal plant, *Withania somnifera* L. cell culture is sustainable source of important medicinal active ingredients. However successful cell culture amenability relies on media composition, cell growth pattern and elicitation strategies. The Glitz (Gli; modified Litvay media) media promisingly supported friable callus induction and maximum growth (15 g biomass) till 56th day of culture but showed lower polyphenols (6.32 mg g-1 DW) and flavonoids (0.15 mg g-1 DW) contents. Further the friable callus was introduced to the liquid Gli media for cell suspension culture establishment. While for increase secondary metabolites synthesis the established cell culture was treated with elicitors; Methyl Jasmonate (MeJa; 5, 10 and 20 µM) and Salicylic Acdi (SA; 50, 100 and 200 µM). The gallic acid standard based total phenolic acid content met the higher value (35.36 mg per g dry cell weight), 3-fold of the control value (10.73 m g^{-1}) in MeJa (20 µM) treated cells. While the higher (3.2 mg g^{-1} dry cell weight) quercetin standard based flavonoid content in comparison to control (0.92 mg g^{-1} DW; 3.5-fold) was observed in SA (100 µM) elicitor treated cell cultures. In parallel DPPH free radical scavenging activity of 60.5% (control: 35.5%) were recorded in elicitors treated cells cultures extracts corroborated to the presence of antioxidant compounds. Based on these findings, it is worth noting that modification in the nutrient media composition significantly improves the *W. somnifera* cells growth (biomass) and secondary metabolism, and this study concept the cellular agriculture for sustainable future in medicinal plants research.

Keywords: biomass; callus; cellular agriculture; elicitors; flavonoids; phenolics; withanolides

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Introduction

In cellular agriculture the optimum plant cell growth (biomass) is crucial, and media composition is the vital determinant of its success because explant types and plant species have different nutritional requirements (Saad and Elshahed, 2012). The several years' research work of tissue culturist produced standard nutrient media, compose of macro (> 0.5 mM) and micro elements (< 0.5 mM), iron, vitamins, carbon source, plant hormones (PGRs) and gelling agent for successful plant cell growth *in-vitro* (Murashige and Skoog, 1962; De Fossard and Bourne, 1977; Trigiano and Gray, 2000; George *et al*., 2008; Saad and Elshahed, 2012). And the slight variation in the composition of these nutrients in media alters the plant cells dynamics, biomass and secondary metabolism (Aisala *et al*., 2023).

The medicinal plant, *Withania somnifera* L. (Solanaceae) commonly called Ashwagandha is extensively used in Ayurvedic recipes (Bano *et al*., 2015*;* Sprengel *et al*., 2025). It is used by the herbalist as decoctions (Kwatha), infusions, ointments (Chrita; medicated ghee or Narayana taila), powder (Churna), and syrups (Arishta; medicated wine). It is distributed globally and exhibits diverse pharmacological activities e.g. antimicrobial, potent antioxidant (Alam *et al*., 2012), and performance enhancing or rejuvenating properties (Bhattacharya and Muruganandam, 2003). *W. somnifera*-has also been reported effective in cardiac (Mohanty *et al*., 2004), cancer (Mohan *et al*., 2004), neurological (Ahmad *et al.*, 2005), immunological (Davis and Kuttan, 2000), liver (Sabina *et al.*, 2013) and inflammatory (Bhattacharya *et al*., 1997) disorders. These medicinal characteristics of *W. somnifera* are attributed mainly to its active constituents such as withanolides (steroidal lactones), polyphenols and flavonoids (the potent antioxidants) (Mirjalili *et al*., 2009).

Globally the cultivable land area is declining rapidly due to various anthropogenic activities which is already been insufficient to meet the food supply to the people. In this scenario the cellular agriculture seems to be the best alternative that offers numerous advantages over conventional and technologically advanced agriculture practices (Baumont *et al.,* 2025). Pakistan is already importing medicinal plants raw material from India while in the Kingdom of Saudi Arabia the limited arable land and costly water resources hinders the medicinal plants cultivation. The increasing food demand and ensuring food security, makes it difficult to substitute medicinal and aromatic plants cultivation in the limited arable fertile land of the Saudi Arabia peninsula. Furthermore, the low germination frequency of Ashwagandha seeds, scarce agriculture knowledge and over exploitation of its natural reservoir have made it an endangered species and makes its cultivation challenging (Chaturvedi *et al.,* 2007*)*. In this case, the promising alternative is plant cell, organ, and tissue culture technology to cultivate it in large bioreactors (cellular agriculture). To achieve this milestone a proper optimization of each tissue culture step like media composition, plant growth regulators and use of specific elicitors are essential to increase biomass and the secondary metabolites production. Pharmaceutical companies and local drug manufacturers depend on the raw material supply, but metabolic inconsistency in the raw material compromises the end products efficacy (Mir *et al.,* 2014; Khan *et al.,* 2015). Plant tissue culture technology offers the only reliable and efficient alternative for obtaining the consistent plant material for effective drug formulations (Skrzypczak-Pietraszek *et al*., 2014). Though in literature numerous studies explores the *W. somnifera* cells and organ cultures for medicinal or nutraceutical ingredients synthesis *in-vitro*. But still viable commercial scale production falls short due to lack of complete information in term of nutrients media requirement, plant hormone selection and growth kinetic studies. Hence this study tries to explore these avenues for adding up to the existing knowledge of plant cell tissue and organ cultures bioprocessing.

In connection with the current study, the withanolides synthesis in *W. somnifera* calli, cells, adventitious and hairy roots, and *in-vitro* shoots cultures are worth mentioning (Roja *et al.,* 1991*;* Banerjee *et al.,* 1994*;* Vitali *et al.,* 1996; Ray *et al.,* 1996*;* Ray and Jha, 2001*;* Sangwan *et al.,* 2007*;* Murthy *et al.,* 2008), but the lower biomass, secondary metabolites quantity falls short in term of industrial feasibility and necessitates to revisit the nutrients recipe for higher cells biomass and medicinal compounds synthesis. The previous studies of Nagella and Murthy tried to optimize the macro and micronutrients, and plant growth hormones for hairy roots growth (biomass) and medicinal compounds (withanolides only) production (Nagella and Murthy, 2010; Nagella and Murthy, 2011). Thus, these studies highlight the role of media salt strength, carbon source and inoculum size for improved biosynthesis of secondary metabolites, and biomass but still the effect of genotype/land race of sourced plant in tissue culture is unavoidable. Additionally, their study presented trade-off in biomass and secondary metabolism; MS media fortified with 0.59 mM NH_4NO_3 supported higher biomass but the addition of 2.09 mM KNO_3 favoured secondary metabolites synthesis in *W. somnifera* roots culture, a two-stage culture system. Therefore, current study aims at the media nutrients optimization for cell culture and explore the possibility of one stage culture system to obtain both biomass and medicinal compounds. This study aimed to optimise the media composition that support biomass as well as secondary metabolism *in-vitro* cultures.

In the present study, the effect of media recipes (varying in plant nutrient composition) on the morphology and growth of calli derived from mature zygotic embryos of *W. somnifera*. In addition to that, *W. somnifera* cell culture was established to study elicitors i.e. salicylic acid (SA) and methyl jasmonate (MeJa) effects on cells growth and medicinally important metabolites synthesis.

Materials and Methods

Zygotic embryos isolation and culture conditions

The isolated *Withania somnifera* L. seeds from fresh collected (Islamabad, Pakistan) berries were rinsed and sterilized according to the protocol of (van der Valk *et al.,* 1992) with some modification. Briefly, isolated seeds were sterilized in sodium hypochlorite (NaOCl) solution (2%, w/v) containing Tween 20 (10 drops per 100 ml) for 24-hr, rinsed in sterilized distilled water (dH_2O), followed by an immersion in 70% (v/v) ethanol for 20 sec and then rinsed with sterilized distilled water (dH_2O). Finally, seeds were further sterilized for about 10 min in H_2O_2 (5%) solution, followed by through sterile water rinse inside the clean bench. The sterilized seeds were de-coated under stereo microscope, zygotic embryos were isolated aseptically and embryos without apparent injury were cultured horizontally on different media (Gli, SOM, MS, and SH) varying in composition (Table 1) in disposable petri-plates (90 mm). There were three replicates, each treatment consisting of five isolated embryos and all cultures were incubated in continuous dark (25 ± 1 °C).

The tested media in this study were prepared according to the described standard procedure (Murashige and Skoog, 1962; Schenk and Hildebrandt, 1972; Litvay *et al.,* 1985) (Table 1). These media varied in composition of major and minor nutrients contents, but the auxin (2,4-D) and sugar concentration remained constant. However, the Glitz, SOM and SH/MS media were solidified with Gelrite (0.3 %), Phytagel (0.18 %) and Agarose (0.8%), respectively for callus initiation and morphology study.

Table 1. Media composition (mg L^{-1}) tested for callus initiation from zygotic embryo of *W. somnifera*

Media components	Gli	SOM	MS	SH
Majors				
KNO_3	950	11685	1900	2500
$MgSO_4.7H_2O$	925	925	180.54	195
KH_2PO_4	170	425	170	-
$CaCl_2.2H_2O$	11	1100	332.02	151
NH4NO_3	825	1380	1650	-
$NaNO_3$	-	-	-	-
$NH_4H_2PO_4$	-	-	-	300

Media components	Gli	SOM	MS	SH
Minors				
$MnSO_4.4H_2O$	21	16.9	16.9	10
H_3BO_3	31	6.2	6.2	5
$ZnSO_4.7H_2O$	43	8.6	8.6	1
KI	4.15	0.83	0.83	1
$CuSO_4.5H_2O$	0.5	0.025	0.025	0.2
$Na_2MoO_4.H_2O$	1.25	0.25	0.25	0.1
$CoCl_2.6H_2O$	0.125	0.025	0.025	0.1
Iron source				
$FeSO_4.7H_2O$	30	27.8	36.70 (Iron EDTA)	19.8
$Na_2EDTA.2H_2O$	40	37.3	-	-
Vitamins				
Thiamine HCl	5	1	0.1	5
Nicotinic acid	5	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5	0.5
Amino acids				
L-Glutamine	500	2250	-	-
Casein hydrolysate	1000	2500	-	-
Inositol	100	5000	100	1000
Glycine	-	2	2.0	-
Sucrose	30,000	30000	30,000	30,000
Agarose	3000 (Gelrite)	1800 (Phytagel)	0.8%	0.8%
2,4-D	1	1	1	1
BAP	0.5	-	-	-
Media pH	5.7	5.7	5.7	5.7

Callus morphology and growth kinetics

To study the effect of media composition on callus induction, isolated zygotic embryos were cultured on different media (Gli, SOM, SH and MS) and callus biomass accumulation was estimated after 6-weeks of culture in triplicates. For callus morphology, forceps touch and visual observations were made for 35 days old callus colour and texture, respectively. For growth kinetics study of callus, 0.5 g of callus inoculum were cultured on the respective media and biomass accumulation was estimated with in intervals of 7-days for 56-days period. For callus induction frequency (CIF) and water content estimation in *W. somnifera* callus the following standard equations were used.

$$CIF = \frac{\text{Number of callus producing explants}}{\text{Total cultured explants in a flask}} \times 100$$

$$Water\ Content = \frac{FW - DW}{FW} \times 100$$

Cell culture growth kinetics and elicitation

To set cell culture, friable and proliferating callus on Gli media (25th day) was selected as inoculum source for cell culture. The 30 g of fresh callus was added to 70 ml liquid (with-out gelling agent) Gli media and stirred for 10 sec in blinder at 120 rpm and was used as stock for cell culture in 250 ml blue cap bottles. The blended callus in liquid medium were poured into volumetric cylinder and were allowed to settle down the suspended small cells aggregates for compact cell volume. The supernatant liquid media was poured off and remaining cells aggregates of ~ 0.5 g fresh weight were introduced into triplicates to blue cap bottles containing 40 ml of fresh Gli liquid media. For growth kinetics (fresh weight and dry weight) study cells from cultures (6-days intervals) were harvested by cultures centrifugation in 50 mL e-Tubes and this was repeated till 54th day of culture. All the experiments were performed in triplicates and cultures were incubated in 24-hr dark conditions at 25 ± 1 °C.

Based on literature survey and preliminary testes *W. somnifera* cells were elicited with salicylic acid (SA) and methyl jasmonate (MeJa) in different concentrations; SA (50, 100 and 200 μM) and MeJa (5, 10 and 20 μM). These elicitors were added to the culture media on day 21 and cells were harvested after 48 days for biomass and secondary metabolites estimation. Fresh and dry weights were recorded in triplicates as g per culture.

Analytical methods

For cells and callus fresh weight and dry weight estimation the described method of Ali and Abbasi, (2013) was used. The obtained dry callus and cells were extracted according to the modified method of Ali *et al*. (2013). Briefly, dried sample (300 mg) was mixed with 70% methanol (2 ml). The mixture was mixed thoroughly by vertexing, followed by sonication for 30 min and then centrifugation for supernatant isolation. The procedure was repeated thrice to ensure complete extraction and isolated supernatant was used immediately for quantitative analysis of secondary metabolites or frozen (4 °C) for later use.

Velioglu *et al*. (1998)described method was used for total phenols content analysis, whereas for the flavonoid content determination in the *W. somnifera* cells extracts Chang *et al.* (2002) described method was used. Gallic acid and quercetin were used as standard for calibration curve deduction. TPC and TFC were expressed as mg g^{-1} dry weight equivalent of gallic acid (GA) and quercetin (QE), respectively.

The 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) quenching potential (% DPPH) of *W. somnifera* cells extracts were studied according to the Abbasi et al. described method *(*Abbasi *et al.,* 2010). Where, 2 mg butylated hydroxyanisole (BHA) in 4 ml methanol with 0.5 ml of DPPH solution was used as positive control for background correction and percent antioxidant activity in samples were calculated according to following formula:

$$\%DPPH\ free\ radical\ quenching\ activity = \left(1 - \frac{AE}{AD}\right) \times 100$$

In the above equation AE is the absorbance of reaction mixture (extract and DPPH solution), while AD denotes the negative control (un-quenched DPPH only) solution (Blank).

HPLC based fingerprinting

Chromatographic analysis was carried out on HPLC-Jasco with LC-Net II system, consisting of a PU-2089 series pump, auto-sampler (AS-2059 Plus) and PDA detector (MDA-2018). Separation of crude methanolic extract was performed on 5 μm Luna C18 (250 x 4.6) column and mobile phase (A = Water Ultra-Pure + 0.05% TFA (pH. 2.6), B = MeOH; HPLC grade) applied in gradient mode at 30 °C. Mobile phase was run using gradient elution at 0, 15, 20 and 23 min of 30, 80, 80 and 30% of solvent-B respectively. The peaks for specific compounds in samples were assigned by diode array detector at 230 nm. For quantification and assigning of respective peaks external standards of chlorogenic acid (100 mg L^{-1}), Moupinamide (25 mg L^{-1}) and withaferin A (146 μMbis) were used. For quantification, series of working solutions of standards were passed through HPLC and peak responses were plotted against injected mass. On the bases of obtained plot, a calibration curve was created and obtained equation were used for quantification of respective compounds in our samples. Furthermore, compounds peaks were identified by comparing the retention times of unknown peaks with the peaks of reference standards in each chromatographic method.

Statistical analysis

This study was completed in three biological repeats per treatment, three independent tissue culture vessels/jars and triplicate data values for each treatment were plotted in excel sheet and statistically analysed for variance (ANOVA) and Tukey's analysis using the Statistix software (v8.2). The callus or cells from respective tissue culture container were treated as respective biological sample for phytochemical data and statistical analysis. For data presentation Origin lab (8.5) software was used to draw visually readable graphs, data point with error bars represents mean values of data and standard error (SE).

Results and Discussions

Effect of media composition on callus growth and secondary metabolites content

The nutrient media composition is crucial in supporting *in-vitro* plant calli, cells or organs growth as well as the secondary metabolism (Rao and Ravishankar, 2002). Therefore, in our study, the response time of explant (zygotic embryo) and callogenesis varied depending on media composition. Indeed, the tested media varied in compositions of $CaCl_2.2H_2O$, KNO_3, minor elements ($MnSO_4.4H_2O$, H_3BO_3, $ZnSO_4.7H_2O$ and KI), amino acids (L-Glutamine and Casein hydrolysate), BAP and gelling agents (Table 1). Out of the tested medias, Glitz (Gli) media was supportive for maximum callus induction from zygotic embryos of *W. somnifera* L. and resulted significantly ($p < 0.05$) maximum biomass accumulation compared to other tested media types (Table 2). Previously, (Hargreaves *et al.,* 2009) used modified Litvay medium (Glitz; Gli) (Litvay *et al.,* 1985) for somatic embryogenesis in zygotic embryos of Christmas trees. Callus of compact and creamy texture with minimum biomass accumulation was observed in MS media (Figure 1C). While callus on SH media was white and friable in appearance with slower growth rate (Figure 1B). Contrary to maximum biomass accumulation on Gli media, maximum total phenolic content was observed in callus obtained on MS media. While maximum flavonoids content was recorded for callus grown on SOM media and was followed by callus grown on MS media (Table 2). Similarly, MS medium was proved to be supportive for secondary metabolite production from *Artemisia absinthium in-vitro* cultures (Ali and Abbasi, 2013; Ali *et al.,* 2016). Previously, (Kim *et al.,* 2004)used immature zygotic embryo of *Catharanthus roseus* and reported embryogenic callus for successful regeneration of plants on MS media.

Table 2. Effect of media composition on *W. somnifera* callus biomass and secondary metabolite content

Media	Callus morphology	FW (g)	DW (g)	Water content %	TPC (mg g^{-1})	TFC (mg g^{-1})
MS	Creamy-compact	3.11 ± 0.115 b	1.7 ± 0.02 b	45.66 b	9.15 ± 0.05 a	0.44 ± 0.015 ab
SH	White-fragile	3.77 ± 0.051 ab	0.68 ± 0.002 c	82.03 a	4.54 ± 0.10 b	0.216 ± 0.003 b
Gli	Green-fragile	14.36 ± 0.17 a	3.44 ± 0.045 a	76.05 a	6.32 ± 0.17 ab	0.154 ± 0.007 b
SOM	Green-compact	4.38 ± 0.05 a	0.97 ± 0.04 c	77.91 a	2.44 ± 0.08 c	0.63 ± 0.006 a

Data were collected after 6 weeks of culture. Values are mean ± SD of three replicates, values with different alphabetical are significant among the columns

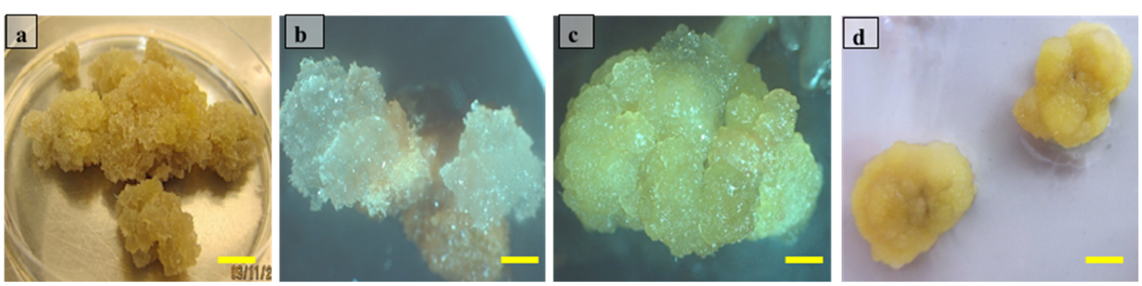

Figure 1. The *W. somnifera* zygotic embryo derived callus morphology effected by media composition: (a) Gli media grown callus is soft and mass of loosely packed rapidly growing cells, and (b) SH media grown callus is white translucent and slow growing cells. While callus (c) on MS media and (d) SOM media are compact in texture

These differences in response of mature zygotic embryos to callus induction and proliferation on different media composition suggest that the amount of calcium chloride, nitrogen source, minor elements and vitamins were crucial for rapid cell growth and division. It has extensively been described in literature that calcium ion acts as transducer of hormonal and environmental signals to the responsive elements of cell metabolism (Evans *et al.,* 1991). Furthermore, Ca^+ modulated plant physiological responses to stress conditions, like salinity, chilling or anoxia (Jaleel *et al.,* 2007). In addition, Ca^+ has been found to be involved in strengthening the cell wall and plant tissues and most importantly it increases the selective uptake of other essential elements from the media as well (Ozaki *et al.,* 2005). Beside the nutrient concentration variation inclusion of BAP into the Gli media might also be the reason for rapid cell division and growth, as hypothesised in cytokinin to auxin ratio hypothesis.

Callus obtained on the respective media were refreshed after 6 weeks and were studied for growth kinetics of 56 days to compare the growth pattern of callus on respective media (Figure 2A, B, C and D).

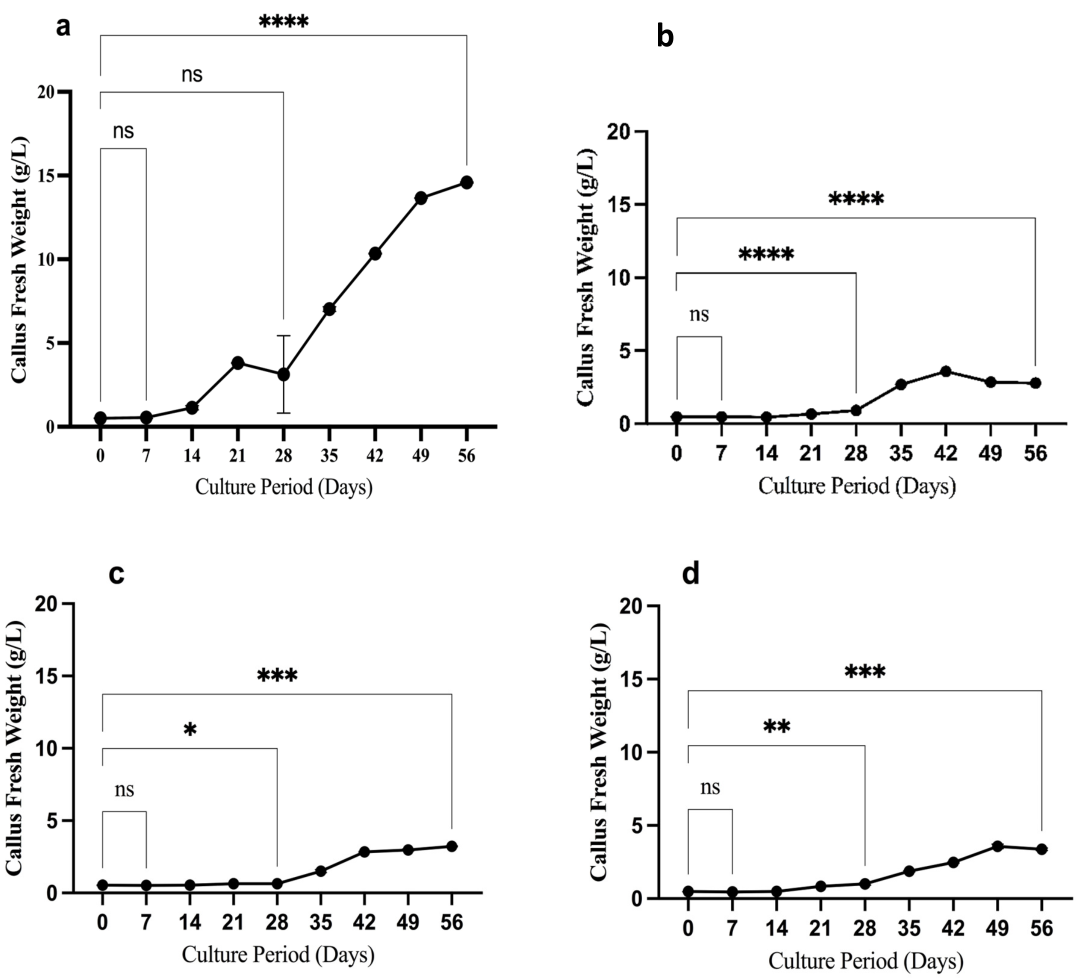

Figure 2. The zygotic embryo derived callus growth kinetics effected by media composition (a) Gli media (b) SH media (c) MS media (d) SOM media

Data points in graphs represent callus fresh weight (FW, g/L) ± SD, recorded in triplicate at 7-days intervals for total 56-days period. The 'ns' in graphs stands for not significant while stars (*) represent significance at * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$

Maximum callus fresh weight with shorter lag phase and yellowish green colour of loosely packed cells were obtained on Gli media at day 56 of the culture (Figure 2A). A prolonged lag phase of 21 days was observed for calli grown on SH, MS and SOM media. Callus grown on SH and SOM media retained their white and yellow green colours respectively throughout the sub-culturing periods (Figure 2B, C and D). While after 42 days of culture, calli on MS media turned brown of crispy texture from creamy green of compact texture. While on SOM and MS media the fresh biomass was reached to its highest at day 49 of the culture and showed a decline afterward (Figure 2C and D). Whereas on SH media growth curve tended to decline after day 42 of the culture (Figure 2D). Friability of callus tissue is highly desirable when establishing cell suspension culture (Akaneme and Eneobong, 2008). It is considered that rapid growth and dividing phenomena determined by active consumption and absorption of essential nutrients has been observed by higher water content in Gli media grown culture (Table 2.) This active consumption of nutrients is linked with lower Ca^+ in Gli-media in comparison to MS, SH or SOM media (Schimansky, 1981; Muhammed *et al.,* 1987;). Alongside this, (Lojewski *et al.,* 2014) observed that media organic constituents also play important role in accumulation of growth promoting essential elements in plant tissues. Therefore the optimal, 100 mg L^{-1} inositol, 500 mg L^{-1} glutamine and 1 g L^{-1} casein hydrolysate in Gli media considered the possible explanation for rapid growth and shorter lag-phase in cell culture of *W. somnifera* (Kadhimi *et al.,* 2014).

Establishment of cell suspension culture and growth kinetics

It is crucial to know the growth phases of cell culture to find the suitable day of elicitation for attaining the maximum biomass as well as secondary metabolites production (Figure 3 and 4).

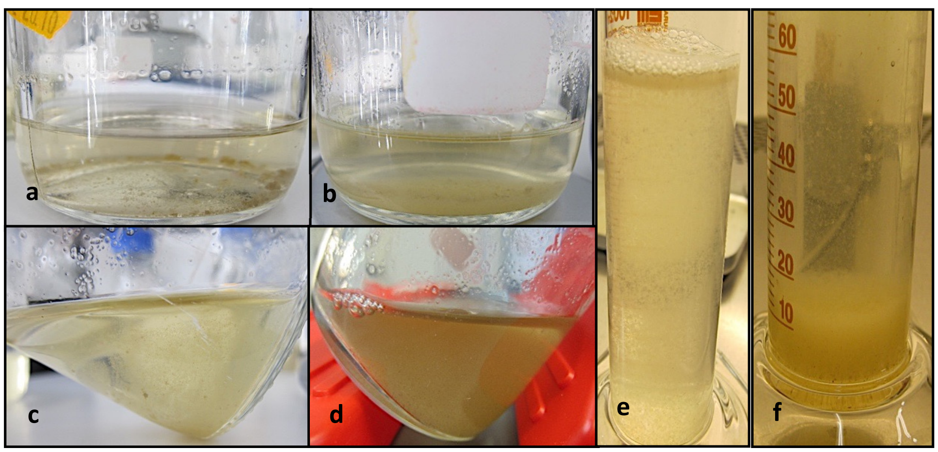

Figure 3. The growth phases of *W. somnifera* L. cell suspension cultures in Gli liquid media; (a) Lag phase, (b) Log phase, (c) Stationary phase, (d) Death phase. While image (e) and (f) suspended cells in liquid culture and Compact cell volume, respectively

The friable green callus with loosely packed cells, obtained on Gli media was selected as source of inoculum for cell suspension culture establishment and blue cap bottles of 250 mL were used to study growth behaviour of cells in agitated liquid media for 54 days (Figure 3). The *W. somnifera* cell culture showed a unique and interesting growth pattern characterised by a short (6-days) lag period followed by a prolonged logarithmic (log) period (lasting for 42-days) on Gli-medium to attain maximum biomass formation (Figure 4A and B). Doubling in fresh weight and dry weight was observed on 12^{th} day of the culture, and cells maximum fresh weight (17.9 g/culture) and dry weight (5.07 g/culture) were reached at 48^{th} day. Furthermore, cell suspension culture was creamy white at log phase and brown in colour at death phase of culture (Figure 3D). The obtained packed cell volume of the cell suspension culture was about 10.3 ml in 100 ml liquid Gli-medium (Figure 3E and F). On the other hand, previously higher biomass was recorded on the 28th day of WS cell culture in MS-liquid media supplemented with picloram and kinetin hormones, and L-glutamine in addition to sucrose (Sivanandhan *et al.,* 2014) probably due to differential mode of action of different hormones.

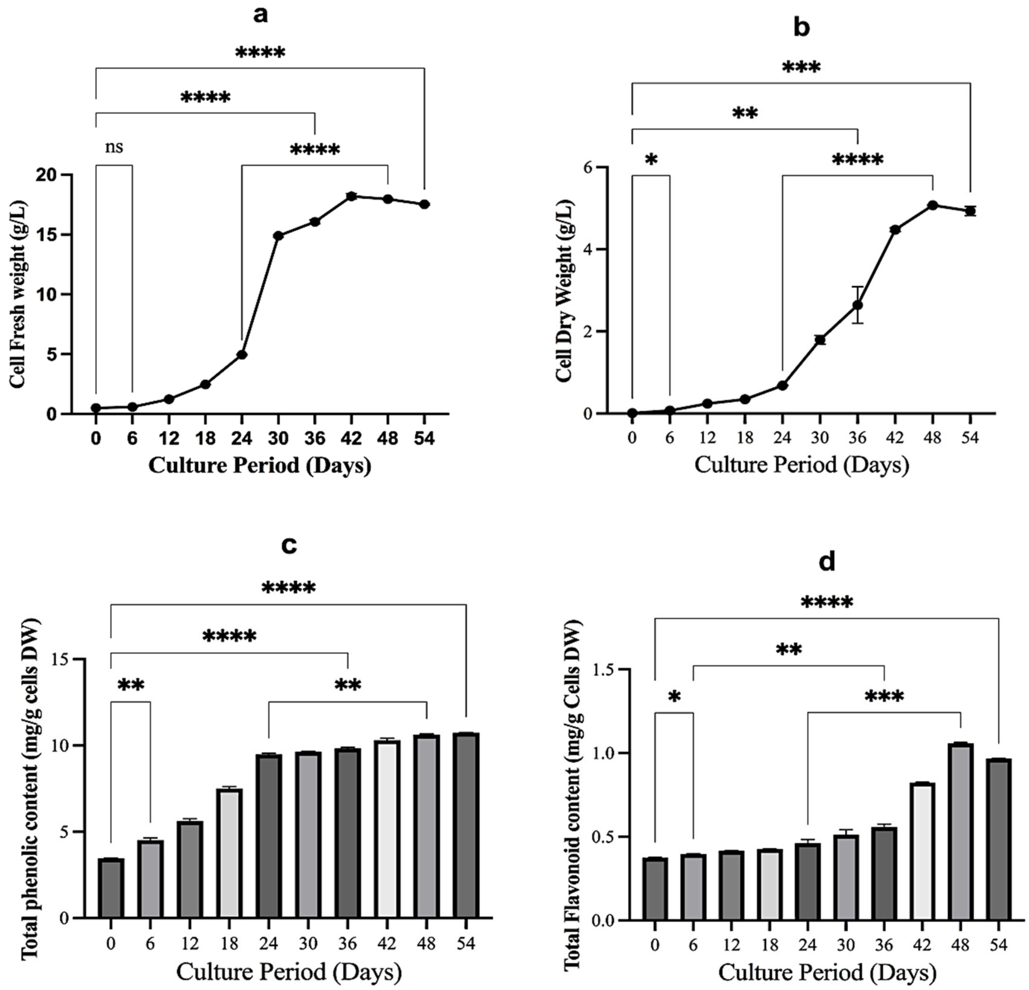

Figure 4. *W. somnifera* cells growth in Gli liquid media; The line graphs (a) and (b) represent cells growth curve (fresh and dry weight) with culture period (6-days interval) and each data point is mean ± SD of three independent repeats. While bar-chart (c) and (d) represent total phenolic and flavonoid contents in cell culture

Data points labelled 'ns' are non-significant (P = 0.062) with respect to data at day zero while stared (**) data is significantly ($p < 0.0001$) different

Antioxidant compounds profile in cell culture

The secondary metabolites synthesis in plant cells is occurring in the exponential growth phase, because cells in lag phase try to adopt the new *in-vitro* conditions (Ouyang *et al.,* 2005). However, we report that the antioxidant compounds (phenolic acids and flavonoids) synthesis shows a growth curve dependent trajectory in the Gli media, probably due to much ideal media nutrient composition to sustain growth and synthesis (Figure 4C and D). It is evident from the literature that certain phenolic compounds are important for plant growth development and their association with plant cell wall explains its growth dependent pattern in *W. somnifera* cell culture (Tanase *et al.,* 2019). On 30th day of the cell culture total poly-phenolics and total flavonoids content were 3-fold and 2-fold respectively, while cell dry weight was 9-fold with respect to dry weight at day zero of the culture. Maximum, 10.6 mg g^{-1} and 1.05 mg g^{-1} total phenolic and flavonoid contents respectively were reached on the 48th day of the WS *in-vitro* cell culture that showed a slight decline beyond this time window. The secondary metabolites (TPC and TFC) accumulation showed an independent pattern to the cell's growth, statistically depicted on heat map chart (Figure 5). Hence proves that rapidly growing cells conserve resources for primary metabolism rather than secondary metabolism, emphasizing the favourable *in-vitro* growth environment.

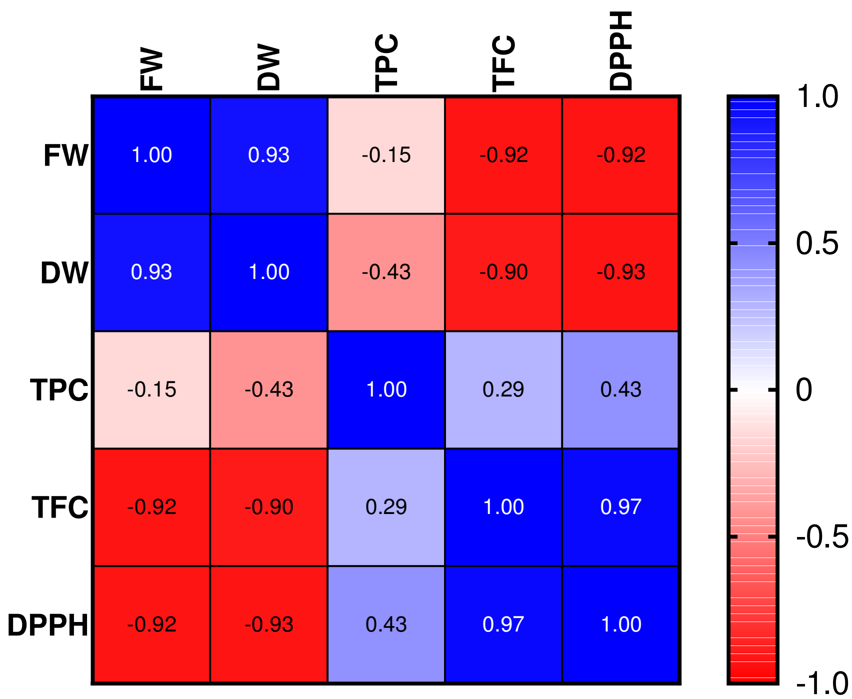

Figure 5. Co-relation of cells biomass with secondary metabolites (phenols and flavonoids) synthesis and DPPH-antioxidant activity

Role of elicitors in-vitro cells growth

Optimization of culture age is one of the critical factors to improve secondary metabolite productions in *in-vitro* cultures Hence, from growth kinetic study we incorporated methyl jasmonate (MeJa) and salicylic acids (SA) to the established *in-vitro* cell suspension of *W. somnifera* on the 21st day (exponential phase) (Figure 6 A and B). While, others suggested 30 days old roots culture optimum age for elicitation (Sivanandhan *et al.,* 2012). However, (Sakunphueak and Panichayupakaranant, 2010) recommended 21 days old culture the optimum age for elicitation. In preliminary investigations we selected 21 days old cell culture as the optimum age for elicitation experiment and observed a significant ($p < 0.05$) variation in cell biomass accumulation after elicitation (Figure 6). The harvested cells fresh weight (FW) of *W. somnifera* varied from 18 g L^{-1} (control) to 10.8 g L^{-1} (~2-fold decrease) and 4.65 g/culture (~ 4-fold decrease) when MeJa (20 µM) and SA (200 µM) respectively were used as elicitors (Figure 6). Similarly, decrease in cell dry weight (DW) was observed and it varied from 5.04 g/culture (control) to 1.74 g and 0.89 g when elicited with MeJa 20 and SA 200, respectively (Figure 6). So, here we concluded that SA was more effective in cell growth reduction. The resulting cells after 54 days of culture were dark brown in colour when treated with SA. For comparison, statistically significant ($p < 0.05$) reduction in biomass accumulation was observed in SA treated cell culture as compared to control and MeJa treated cells.

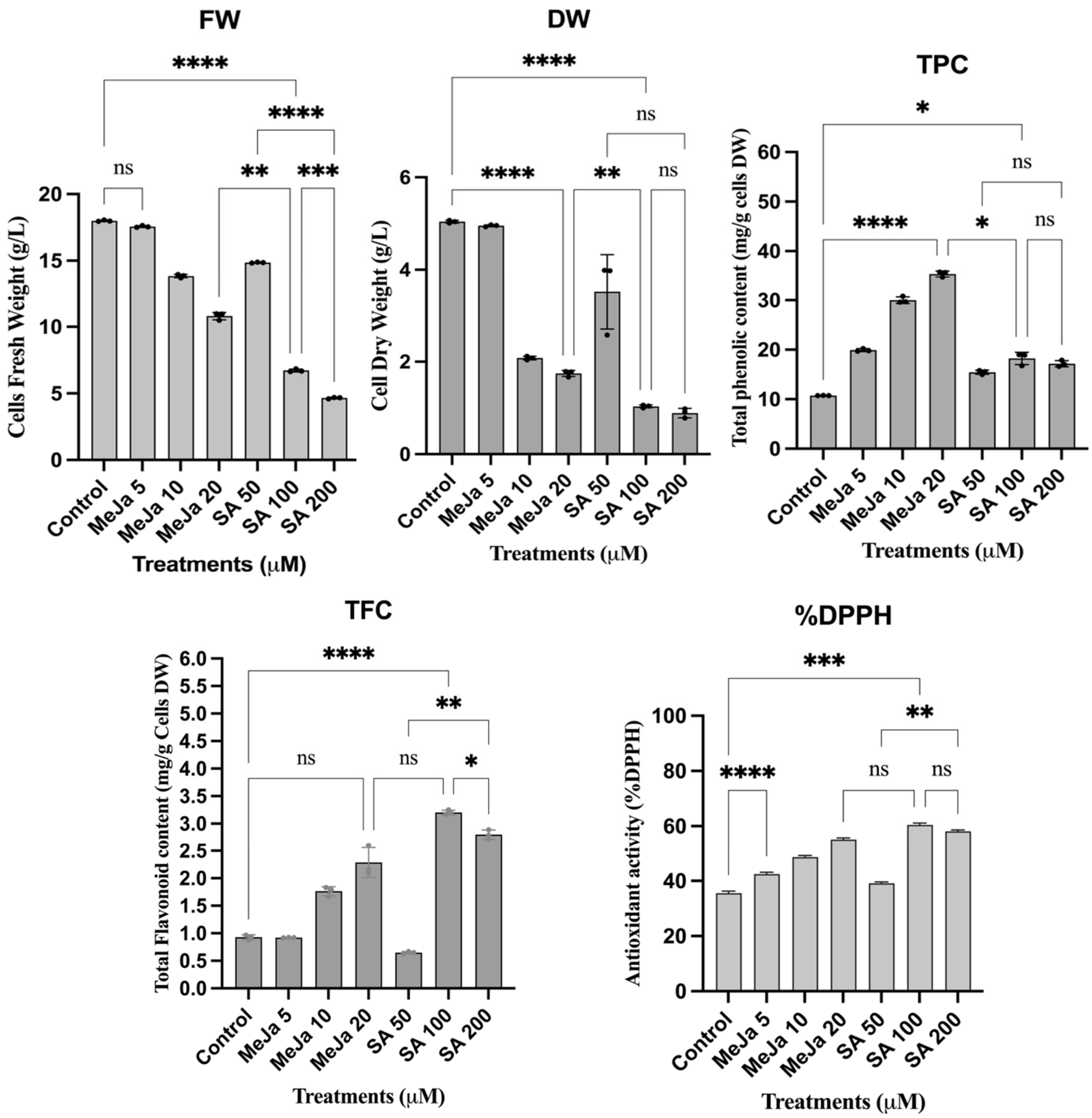

Figure 6. The elicitors, methyl jasmonate (MeJa; 5, 10 and 20 μM) and salicylic acid (SA; 50, 100 and 200 μM) treated *W. somnifera* cell culture shows varying response in term of biomass (fresh and dry weight, g L^{-1}) accumulation, secondary metabolites (total phenolic and flavonoid content, mg g^{-1} DW) and antioxidant activity

Each bar with the SD bar represents mean ± SD of three replicates and star bars are statistical significant to control value while 'ns' is not significant value

Rao *et al.* (1997) reported that exogenous application of SA, enhanced the lipid peroxidation and caused oxidative damages to *Arabidopsis thaliana* that ultimately caused reduction in biomass accumulation and similar observations were in tobacco and *Taxus chinensis* cultures (Yu *et al.,* 2002; Harish *et al.,* 2024). Similarly, Xu *et al.* (2015) found significant reduction in cells growth of *Vitis vinifera* L. when elicited with higher concentrations of elicitors. However, Ali *et al.* (2015) reported 3-fold increase in MeJa treated cells dry biomass with shorter log phase of *Artemisia absinthium* L.

Poly-phenolics and flavonoids synthesis in elicitors treated cultures

Plant Cells farming (cellular agriculture) is an attractive complementary option since plant cell are totipotent, able to express whole genome to produce useful metabolites and can be manipulated for maximum production by employing suitable elicitor (Signalling molecules) at suitable concentration. Where elicitors are signalling molecules which mediates the plant defence pathways that leads plants to produce various protecting compounds such as phenolics, flavonoids and other low molecular weight metabolites (Yan *et al.,* 2005; Ali *et al.,* 2006). Based on literature, the signalling molecules (SA and MeJa) are considered potent extracellular elicitors for enhance synthesis of important secondary molecules in medicinal plants (i.e. *W. somnifera*) cells cultures (Jeyasri *et al.,* 2023). In corroboration to these findings, our study finds secondary metabolites differential stimulatory responses to the type and concentration of the elicitors used. For instance, the SA treated cell cultures at concentration of 100 µM increased the total flavonoid content by 3.5-fold, significantly higher than the control cultures. While, with respect to control the total phenolic content reached to its maximum value of 35.36 mg GAE/g DW when MeJa (20 µM) was used as elicitor (Figure 6). These results inferred that SA elicited the phenolic compounds production while in MeJa treated cultures favoured total flavonoid production. This difference could be due to the differential modes of actions of these elicitors in plant cells metabolic pathways. Previously, SA acid treatment activated PAL enzymes in *Salvia miltiorrhiza* callus that enhanced the salvianolic acid B and caffeic acid synthesis (Dong *et al.,* 2010). While, (Kenmotsu *et al.,* 2013) observed transcriptional activation of farnesyl diphosphate synthase in MeJa treated cell culture of *Aquilaria microcarpa*. So, from this study we propose the combinatorial effect of SA and MeJa on medicinally important metabolites (flavonoids and phenols) production.

Additionally, the reactive oxygen species (ROS) scavenging potential in *W. somnifera* cells extracts was confirmed on DPPH$^{\cdot}$ assay and results were supported by the phenols and flavonoids content values. Interestingly the DPPH reactive species quenching potential was dependent on total flavonoid content and independent on cell biomass. Highest antioxidant activity (60.5%) and maximum flavonoid content (3.2 mg QE/g DW) were recorded in cell culture treated with SA (100 µM) (Figure 6). A decline in antioxidant activity was observed with further increase of SA concentration. Among MeJa treated cell culture extracts contributed to the DPPH$^{\cdot}$ free radical scavenging activity in range of 42 to 55%. Significantly lower antioxidant activity (35%) was observed in control treatment (Figure 6).

Effect of elicitors on chlorogenic acid and withanolide content

Among different tested concentrations of MeJa and SA, 20 µM MeJa addition into growth media resulted an enhanced production of chlorogenic acid (24.78 mg g^{-1} DW; 6-fold), moupinamide (18.78 mg g^{-1} DW; 4-fold), withanoside IV (11.46 mg g^{-1} DW; 2.3-fold) and withaferine A (11.19 mg g^{-1} DW; 2-fold) when compared with control (Figure 7). This could be due to the (Gómez *et al.,* 2010) hypothesis that agrees with altered allocation of resources in plants to buffer the stress by increasing plant cell growth and survival. We observed less significant ($p < 0.05$) difference among control and cells treated with 50 µM SA. While an increase was observed when SA concentration was doubled and beyond this decrease in content of detected compounds were observe. The results suggested that the cells treated with elicitors had different abilities to produce secondary metabolites (Koul and Mallubhotla, 2020). Hence, cell treated with MeJa (20 µM) resulted maximum integrated peak area, followed by MeJa (10 µM) and SA (100 µM) (Figure 7). The increased metabolites may indicate that transcript levels of genes involved in the secondary biosynthesis has been induced by MeJa (Gadzovska *et al.,* 2007).

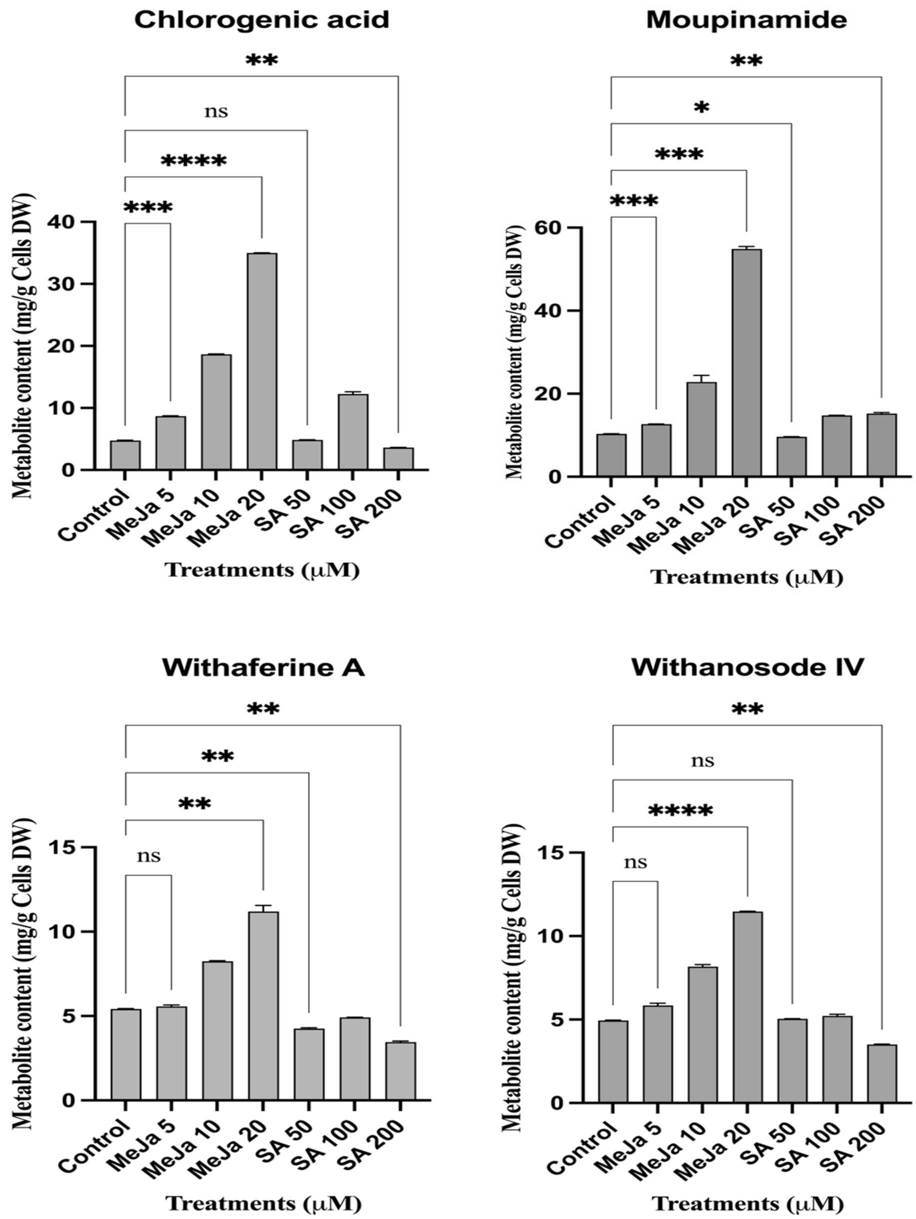

Figure 7. Effect of methyl jasmonate (MeJa) and salicylic acid (SA) with respect to control on chlorogenic acid, moupinamide, withanoside IV and withaferin A contents (mg g^{-1} DW) in cell suspension cultures of *W. somnifera*

Each bar in charts represents mean ± SD of three replicates, steric data is significantly deferent while bar labelled as 'ns' is not significant with respected to un-treated (control) cell cultures

MeJA and SA have been long known to contribute to defence via signalling mechanism to counter plant pathogens or other environmental stresses (Farhadi *et al.,* 2020). Our results hits to the possibility of these hormones to play a pivotal role in reprogramming plant development. (Sánchez-Sampedro *et al.,* 2005) reported exposure of *Silybum marianum* cells to extra cellular elicitor (MeJa) enhanced the activity of chalcone synthase (i.e., an enzyme in silymarin biosynthesis). Our results are in consistent with previous reports demonstrating higher withanolides (withaferin, withanone and withanolide) content in *W. somnifera* hairy roots exposed to MeJa treatment (Sivanandhan *et al.,* 2013). Similarly another study described improve total phenolic content in MeJa treated banana than SA (Mendoza *et al*. 2018). Moreover, a significant increase of phenolics, flavonoids and acacetin contents was observed in *S. kakudensis* cells exposed to MeJa elicitors (Manivannan *et al.,* 2016). The simulating effect of MeJa (elicitors) in plant defence system activate ROS scavenging cascade inside the cells that enhances secondary metabolism to synthesis antioxidant compounds and other active ingredients (Lamb and Dixon, 1997; Zhang and Xing, 2008).

Conclusion

This research describes the friable callus induction from *Withania somnifera* L. zygotic embryo in Gli media - a novel in term of nutrients composition - effective to support rapid cell growth even in liquid cultures. The 21-days old cells in mother media (Gli), treated with MeJa and SA differentially induced the secondary metabolites production. The SA (100 µM) treated cells fabricated flavonoids production while MeJa (20 µM) induced phenolic compounds production an interesting finding for future research to engineer *W. somnifera* pathways for industrial applications. The finding of suitable nutrient media and elicitors unveils the important bottle neck in *W. somnifera* cell culture and bioindustries research, and clears the path for further research to cultivate *W. somnifera* cells sustainably for future industrial applications.

Authors' Contributions

Conceptualization: MA; Writing - original draft: MA; Supervision: MA; Formal analysis: AAKK; Methodology: AAKK; Data Curation: ZY; Writing - review & editing: ZY; Writing - review & editing: KALR; Resources: AALK; Visualization: AALK; Writing - review and editing: ZURM; Visualization: ZURM; Validation: ZURM.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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