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Amelioration of withaferin-A Accumulation in 2,4-D-induced Calli of *Withania somnifera* (L.) Dunal

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Key words: Ashwagandha, Friable callus, Principal component analysis, Relative water content, UPGMA clustering

Abstract

The present study investigated the impact of varying doses of 2,4-dichlorophenoxyacetic acid (2,4-D) on friable callus induction and withaferin-A (WA) accumulation in *Withania somnifera*, popular as 'Indian ginseng'. Friable calli cultures were established using variable doses of 2,4-D (1.0-5.0 mg/l). The 5.0 mg/l 2,4-D significantly enhanced callusing percentage (100%), fresh weight (795.0 mg), dry weight (DW) (99.0 mg), relative water content (709.0%), as well as WA content (0.043 mg/100 g DW), demonstrating its superiority over other treatments. Pearson's correlation analysis revealed strong positive associations among callus biomass traits and WA content, whereas days to callus induction showed weak correlations. Multivariate approaches, including correlation, principal component analysis and unweighted pair-group method with arithmetic mean clustering, effectively distinguished the 5.0 mg/l treatment as optimal. Overall, the findings establish a high-efficiency *in vitro* protocol for biomass and WA production in *W. somnifera* through friable callogenesis, offering valuable insights for large-scale phytochemical production and plant tissue culture applications.

Introduction

Withania somnifera (L.) Dunal, commonly known as Indian ginseng or Ashwagandha, belonging to the family Solanaceae, is a perennial medicinal shrub naturally distributed in semi-arid and arid parts of Africa, the Middle East, and throughout India (Kaur et al. 2021a). Renowned for its long-standing use in traditional medicine, it is now globally recognized for its pharmacological properties, including immunomodulatory, neuroprotective, anti-inflammatory and adaptogenic effects. These bioactivities are largely due to its rich content of alkaloids and steroidal lactones called withanolides, predominantly found in the leaves and roots (Singh et al. 2023). Of the ~40 known

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withanolides, withaferin-A (WA) is the most studied for its strong anti-oncogenic properties, inducing apoptosis and suppressing metastasis in pancreatic, lung, and breast cancers (Kaur et al. 2021b, Kumar et al. 2023, Singh et al. 2023).

Friable callus refers to a soft, pale, and loosely textured mass of undifferentiated plant cells that readily disintegrates into small cell clusters or individual cells when subjected to gentle mechanical agitation. It is typically induced on culture media enriched with high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) alone, or mixed with cytokinin. Unlike compact or hard callus, which is dense and rigid, friable callus has a granular structure that facilitates its dispersion in liquid media, making it particularly suitable for downstream applications. Friable callus plays a crucial role in various fields of plant tissue culture and biotechnology. One of its most significant applications is in the establishment of cell suspension cultures, which are essential for the mass production of valuable secondary metabolites such as alkaloids, flavonoids, and withanolides. These cultures provide a controlled and sterile system for studying plant metabolic pathways and are extensively used in the pharmaceutical and nutraceutical industries (Mirjalili and Esmaeili 2022). Despite having low regeneration capacity when compared to compact callus, friable callus also serves as an excellent starting material for somatic embryogenesis, genetic transformation and protoplast isolation, allowing for efficient gene transfer and plant regeneration. Additionally, the uniform nature of friable callus is favored in molecular studies, mutagenesis, and synthetic seed technology. However, its effectiveness depends on species-specific responses and careful optimization of culture conditions to balance growth, viability, and regeneration potential. Despite many advantages, there are few studies that focus on friable callogenesis and WA production; hence, the aim of this study is to establish a short protocol to induce friable calli in *W. somnifera* to enhance WA accumulation.

Materials and Methods

Shoot tips of *W. somnifera* (var. Gujarat Anand Ashwagandha-1) were harvested from two-month-old mother plants. These 2-3 cm long shoot tips were rinsed with sterile water and then treated with disinfectants following Bandyopadhyay et al. (2025). Shoot tips were then trimmed to approximately 2 cm and cultured *in vitro* on MS medium to initiate the multiple shoots. Once established successfully, from one-month-old plantlets, internodes were chopped to about 2 cm in size and inoculated in the callogenesis media.

MS medium with added $CaCl_2$, 0.7% (*w/v*) agar, vitamins, 0.02% (*w/v*) myo-inositol and 3% (*w/v*) sucrose was used as the basal medium in which diverse doses (1.0, 2.0, 3.0, 4.0, and 5.0 mg/l) of 2,4-D were supplemented and pH of the media was attuned to 5.7 before autoclaving at 121°C and 1.1 kg/cm² for 20 min. The cultures were incubated under sterile conditions at 60% relative humidity, a temperature of 23 ± 2°C and a 16 h photoperiod inside a growth room. Photoperiod was provided by cool white Philips LED tube-lights, delivering an average photosynthetic photon flux density of 50 µmol/m²/s.

Internodal explants from multiple shoot culture were inoculated in MS medium supplemented with the abovementioned doses of 2,4-D alongside a 'control' (without 2,4-D). The cultures were monitored over a period of four weeks to determine the most effective concentration of this plant growth regulator (PGR). Following callogenesis, data were collected on days to callus initiation (DCI), percentage of callusing (%), fresh weight (FW) and dry weight (DW) of the callus (in mg), and relative water content (RWC) (%) [{(FW–DW)/DW} × 100]. Additionally, WA was extracted from all callus samples and quantified subsequently.

Friable calli of different stages, obtained from the callogenesis medium, were carefully taken out from the culture tubes, the adhered media were precisely removed and the calli were fixed in 5% (*v/v*) formalin solution (having 37-41% formaldehyde) for seven days to preserve cellular structures. Subsequently, the samples underwent a step-by-step dehydration process using graded alcohols, after which they were embedded in paraffin wax blocks. Ultra-slim sections were obtained using a microtome and floated in a warm water bath to flatten the tissue slices. These sections were then stained using haematoxylin and eosin stain to visualize cellular components. Finally, the stained tissue slices were mounted onto glass slides, covered with coverslips, and prepared for microscopic examination.

The WA contents in calli were quantified using a high-performance liquid chromatography (HPLC) system (Waters™, Milford, USA). Calli samples from all the treatments were dried at 50°C for approximately two days. Once fully dried, 10 mg of tissue was finely ground and homogenized in 1.0 ml of HPLC-grade methanol. The homogenate was briefly vortexed, followed by sonication for 45 min and centrifugation at 5000 × g for 5 min. The resulting supernatant was filtered through a 0.22 μm syringe filter (Sartorius) for analysis. For standard preparation, 1.0 mg of pure WA (Sigma-Aldrich, USA) was dissolved in 1.0 ml of HPLC-grade methanol, as described by Sharada et al. (2007). Quantification was performed using an HPLC system equipped with a reverse-phase C18 column (5.0 µm, 4.6 mm × 250 mm). An isocratic elution was employed using a mobile phase consisting of potassium dihydrogen phosphate buffer with pH adjusted to 3.2 with orthophosphoric acid (phase A) and acetonitrile (phase B) in a 65:35 (*v/v*) ratio. Each run lasted for 15 min, and detection of WA was performed at 227 nm using a UV-Vis detector. The flow rate and injection volume were set at 1.0 ml/min and 10 µl, respectively, following the method of Kaur et al. (2021a).

The study was performed employing a completely randomized design with three replications. Data were homogenized and analyzed using one-way ANOVA, and further evaluated post-hoc through Duncan's multiple range test (Duncan 1955) in SPSS (v26.0, SPSS Inc., Chicago, IL, USA). Graphical analyses, including correlation, principal component analysis (PCA), and unweighted pair-group method with arithmetic mean (UPGMA) clustering, were done using PAST (v4.02) (Hammer et al. 2001).

Result and Discussion

The number of days required for callus induction decreased significantly with increasing 2,4-D concentration. While no callus (Fig. 1a) was induced in the control, the maximum delay (19.3 days) in callus induction was seen at 1.0 mg/l 2,4-D (Table 1). The quickest (7.7 days) callus induction occurred at 5.0 mg/l, indicating that higher auxin levels accelerate callus formation.

Table 1. Effects of 2,4-D (supplemented in MS medium) on callus induction, proliferation, and WA accumulation in *W. somnifera* (at four-week growth stage).

2,4-D (mg/l)	DCI	Callusing percentage (%)	Callus FW (mg)	Callus DW (mg)	RWC (%)	WA content (mg/100 g DW)
0	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0f	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0d
1.0	19.3 ± 0.9a	13.3 ± 3.3d	56.0 ± 5.0e	19.3 ± 4.1c	206.0 ± 41.4c	0.006 ± 0.001cd
2.0	14.7 ± 0.3b	45.0 ± 2.9c	91.0 ± 3.5d	31.3 ± 0.9c	190.3 ± 3.8c	0.008 ± 0.002c
3.0	12.0 ± 0.6c	91.7 ± 1.7b	183.0 ± 6.6c	27.0 ± 3.6c	598.4 ± 77.2a	0.017 ± 0.003b
4.0	12.0 ± 0.6c	96.7 ± 1.7ab	250.3 ± 16.5b	48.3 ± 5.5b	423.4 ± 26.8b	0.020 ± 0.003b
5.0	7.7 ± 0.3d	100.0 ± 0.0a	795.0 ± 18.1a	99.0 ± 7.2a	709.0 ± 40.6a	0.043 ± 0.003a

Data stand for mean ± standard error of 3 replicates per treatment. Data in each *column* followed by different *letters* are significantly different according to Duncan's multiple range test (Duncan 1955) at P <0.05.

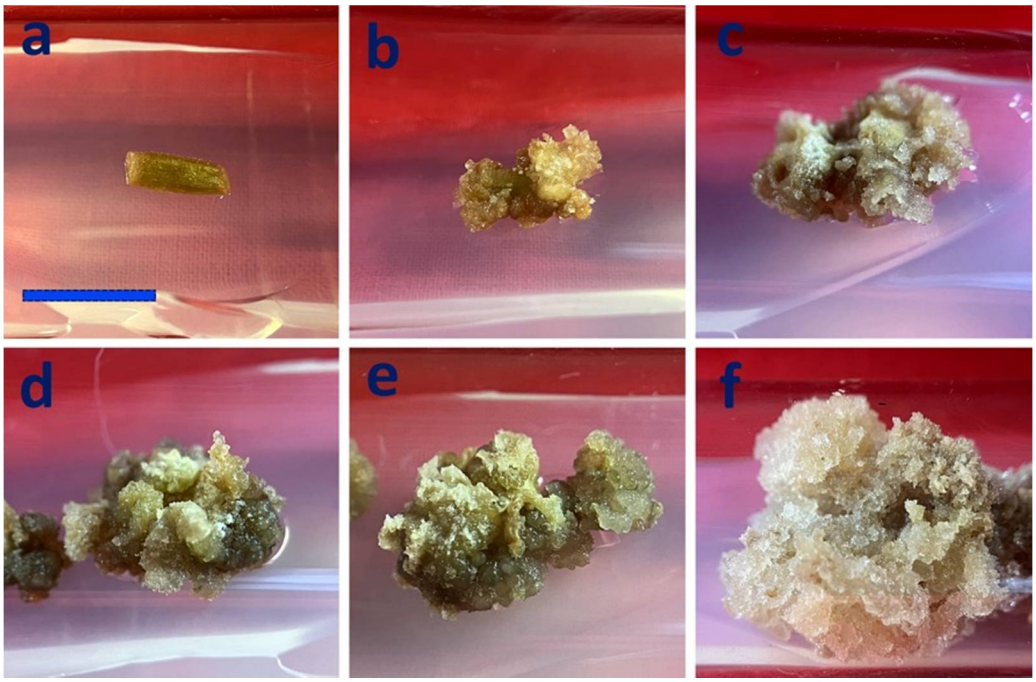

Fig. 1. Differential response of *W. somnifera* internode explants to callus induction and proliferation (at four-week growth stage) in MS medium supplemented with: (a) 0 mg/l 2,4-D (Control), (b) 1.0 mg/l 2,4-D, (c) 2.0 mg/l 2,4-D, (d) 3.0 mg/l 2,4-D, (e) 4.0 mg/l 2,4-D and (f) 5.0 mg/l 2,4-D. (*Bar* = 25 mm).

Callus induction percentage increased progressively with rising 2,4-D concentrations. It ranged from nothing in control to 100% at 5.0 mg/l 2,4-D, showing a strong dose-dependent response. Notably, a sharp increase in callusing was observed beyond 2.0 mg/l, peaking at 5.0 mg/l 2,4-D. Friable callus formed in 1.0-4.0 mg/l 2,4-D had a greenish tint (Fig. 1b-e) probably due to the presence of an underneath regenerative compact cell layer, while the callus generated in 5.0 mg/l 2,4-D was pure white indicating cent percent friability (Fig. 1f). Histological analysis also confirmed that the callus obtained from 5.0 mg/l was 100% friable in nature (Fig. 2a), whereas the same obtained from 3.0 mg/l treatment had a layer of compact cells under the friable section (Fig. 2b). The histology study also identified the stacking of loose irregular shaped cells to be highly friable (Fig. 2c).

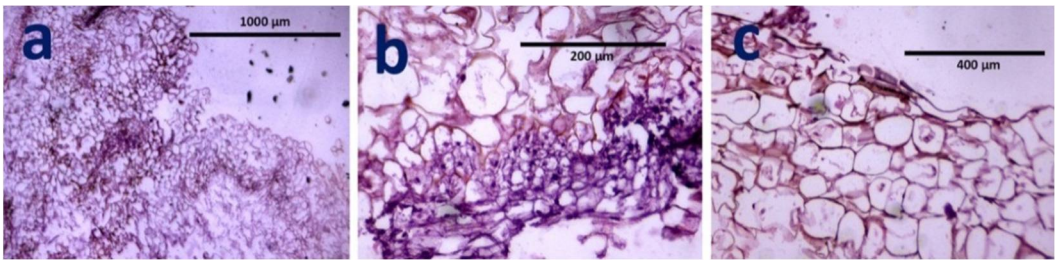

Fig. 2. Histological analysis of *W. somnifera* calli induced from 2,4-D-supplemented MS medium: (a) irregular-shaped edge of the friable callus obtained from 5.0 mg/l 2,4-D, (b) friable cells emerging from the inner compact cell layer (callus obtained from 3.0 mg/l 2,4-D) and (c) loose association of large cells of friable callus obtained from 5.0 mg/l 2,4-D.

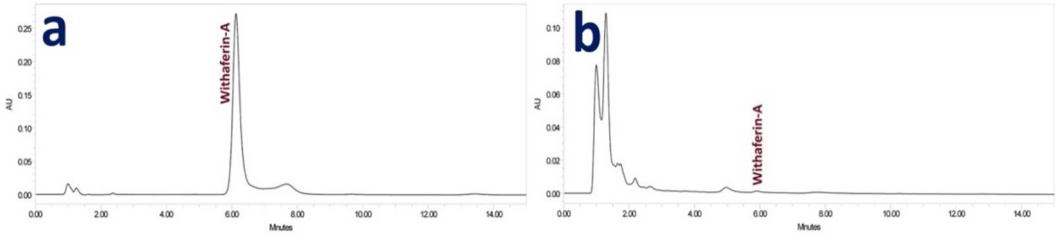

Fig. 3. Representative HPLC chromatograms of (a) WA standard and (b) *W. somnifera* callus methanol extracts from 5.0 mg/l 2,4-D supplemented MS medium (x-axis represents retention time, y-axis represents absorbance units).

FW of the callus also increased with increasing 2,4-D levels (1.0-5.0 mg/l). From no growth in control, FW augmented significantly across treatments, reaching a maximum (795.0 mg) at 5.0 mg/l 2,4-D. This reflects enhanced callus proliferation at higher auxin concentrations, especially above 3.0 mg/l. DW followed a similar trend to FW, ranging from 0 mg in the control to a maximum of 99.0 mg at 5.0 mg/l (Table 1). The substantial rise in DW at higher 2,4-D levels (4.0-5.0 mg/l) highlights the improved tissue mass accumulation under optimal growth regulator conditions. RWC increased with rising 2,4-

D concentrations, indicating better water retention and hydration status of the callus. From 0% in control, RWC reached a maximum (709.0%) at 5.0 mg/l. The spike in RWC at 3.0 mg/l and above suggests that metabolically active, hydrated tissues form at higher auxin doses.

WA accumulation showed a remarkable increase with increasing callus biomass. The highest concentration of 2,4-D promoted the maximum synthesis of WA when compared with the standard (Fig. 3a). From barely detectable levels in control, the WA content rose steadily, reaching 0.043 mg/100 g DW of callus tissue at 5.0 mg/l 2,4-D (Fig. 3b), confirming a strong positive relationship between callus quality and metabolite production.

The present study reveals that progressive increases in 2,4-D concentration (1.0-5.0 mg/l) significantly improved callus induction rate, biomass production (both FW and DW), RWC, and WA accumulation in *W. somnifera*. Notably, 5.0 mg/l 2,4-D yielded the best results across all parameters, highlighting it as the most effective dose for promoting *in vitro* callus development and secondary metabolite biosynthesis. Comparable efficacy of 2,4-D has been previously reported by Rani et al. (2016), where 2.0 mg/l 2,4-D, along with 0.5 mg/l Kn showed callogenesis in *W. somnifera*. Similarly, 2.0 mg/l 2,4-D combined with 0.2 mg/l Kn was reported to induce callogenesis in *W. somnifera* (Udayakumar et al. 2014). In other plant species, 2,4-D has been successfully employed to induce friable callus in *Calotropis gigantea* (Muthi'ah et al. 2023) and *Clitoria ternatea* var. *albiflora* (Zakaria et al. 2024).

The correlation matrix (Fig. 4a) illustrates the interrelationships among various callus-associated traits in *W. somnifera* exposed to different concentrations (1.0-5.0 mg/l) of 2,4-D. Notably, strong positive correlations were observed among callusing percentage, FW, DW, RWC and WA content, suggesting that a higher callus induction percentage is closely linked with greater biomass production and secondary metabolite accumulation. These variables appear to act together, indicating a possible synergistic response under suitable hormonal conditions. Conversely, DCI exhibited minimal or no significant correlation with the other traits, indicating that the timing of callus initiation has limited influence on biomass production or WA yield. Overall, the matrix highlights that optimizing callus development and associated physiological traits can substantially enhance WA production *in vitro*. Similarly, in a recent study, Gonzalez et al. (2024) used correlation analysis to explore the relationships between multiple biochemical parameters in *Simmondsia chinenesis* under salt stress conditions.

PCA biplot (Fig. 4b) depicts the variation in callus-related traits of *W. somnifera* under different 2,4-D treatments. The first principal component (Component 1) accounts for 90.5% of the total variance, while the second (Component 2) explains 9.3%, together representing 99.8% of the total variability, indicating a highly informative model. The treatments are distributed across the plot based on their association with six key traits: DCI, callusing percentage, FW, DW, RWC and WA content, represented by green vectors. The control and low 2,4-D concentrations (1.0 and 2.0 mg/l) cluster on the

negative side of Component 1, indicating a weaker response in all measured traits. The 2.0 mg/l treatment, situated near the origin and closely aligned with WA content, DW, DCI and callusing percentage, indicates a balanced impact on both biomass and metabolite accumulation. 3.0 mg/l 2,4-D treatment appears in the upper right quadrant, aligning strongly with RWC. Notably, the 5.0 mg/l 2,4-D treatment is positioned far on the positive side of Component 1 and is most closely associated with FW, indicating maximum callus biomass accumulation. In conclusion, 5.0 mg/l 2,4-D emerges as the most effective concentration for simultaneously enhancing callus quality and WA production, favouring both bulk biomass and biochemical productivity. For years, PCA has been a reliable analytical tool for researchers to compare the effect of various PGR combinations on specific growth parameters due to its ability to visualize datasets with a greater clarity. Recently, it has been employed in studies across multiple plant species, including *Dendrobium* hybrid Yuki White (Subrahmanyeswari et al. 2022), *Cerastium candidissimum* (Bertsouklis and Tsopela 2023) and *W. somnifera* (Bandyopadhyay et al. 2025).

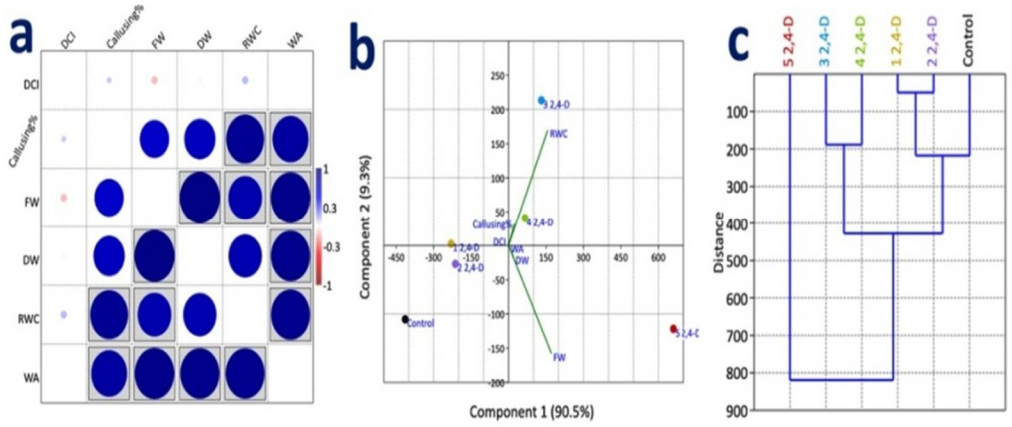

Fig. 4. Effect of 2,4-D supplementation (1.0-5.0 mg/l) in comparison to control: (a) correlations among growth traits ('boxed' represent significance at P <0.05), (b) PCA (scattered plot/bi-plot) and (c) UPGMA clustering based on Euclidean distance matrix elucidating the association of treatments.

The dendrogram (Fig. 4c) represents a hierarchical cluster analysis based on the response of *W. somnifera* to varying concentrations of 2,4-D. It shows three major clusters: the first one comprises treatments including control, 1.0 and 2.0 mg/l 2,4-D, indicating a high degree of similarity in their effects on callus traits and metabolite production. The second cluster includes 3.0 and 4.0 mg/l 2,4-D, suggesting moderate similarity among them. The 5.0 mg/l treatment stands alone, forming a distinct outgroup, which reflects its significantly different and enhanced behaviour. This clustering pattern highlights the dose-dependent influence of 2,4-D on *in vitro* responses, with higher concentrations (particularly 3.0-5.0 mg/l) producing closely related and superior outcomes in terms of callus development and biochemical parameters. Previous studies have demonstrated

the impact of various growth-promoting compounds on *in vitro* morphological growth parameters in *W. coagulans* Dunal using UPGMA clustering (Tripathi et al. 2018). In another study, UPGMA cluster analysis and heat map-based cluster analysis were used together to visualize the same in *Cymbopogon schoenanthus* subsp. *proximus* (Abdelsalam et al. 2022).

The present study highlights the significant influence of 2,4-D concentration on callus induction and a vital secondary metabolite production in *W. somnifera*. Among the tested treatments, 5.0 mg/l 2,4-D demonstrated the highest efficiency in enhancing callusing percentage, biomass accumulation (FW and DW), RWC, and WA production. PCA and correlation matrix analyses revealed strong positive relationships among these physiological and biochemical traits, indicating a synergistic response under optimal hormonal conditions. UPGMA clustering and PCA further distinguished 5.0 mg/l as the most effective treatment, clearly separating it from the lower concentrations and the control. The minimal correlation of DCI with other parameters suggests that rapid initiation alone does not ensure better biomass or metabolite output. Overall, the findings support the potential of high-dose 2,4-D in promoting efficient *in vitro* propagation and phytochemical accumulation. This optimized protocol can contribute to enhanced secondary metabolite yields in medicinal plant tissue culture systems.

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