



Research article

Altered biomass allocation and quality improvement in roots of Indian ginseng *Withania somnifera* Dunal. Linn. through physiological interventionsKuldeepsingh A. Kalariya^{a,*}, Narendra A. Gajbhiye^a, Dipal Minipara^a, Parmeshwar L. Saran^a, Sushil Kumar^b, Vanrajsinh Solanki^c, Susheel Singh^c, Prince Choyal^a, Ponnuchamy Manivel^a^a ICAR-Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand Gujarat, India^b Department of Biotechnology, Anand Agricultural University, Anand Gujarat, India^c Food Quality Testing Laboratory, Navsari Agricultural University, Navsari Gujarat India

ARTICLE INFO

Keywords:

Biomass partitioning
Gene expression
Residue analysis
W. somnifera
Withanoloides
Horticulture
Plant physiology
Phytochemistry
Root
Metabolite
Chromatography
Plant biology
Agriculture

ABSTRACT

Ashwagandha (*W. somnifera* Dunal. Linn.), known as Indian ginseng, contains three major bioactive compounds, withaferin-A (WA), 12-deoxywithastramonolide (WO) and withanolide A (WD). In a field experiment, the impacts of foliar application of growth retardants/promoters was assessed with respect to biomass allocation pattern and major withanolide content at different phenological stages in *W. somnifera*. Biomass accumulation pattern showed that foliar application of 500 mg l⁻¹ ethrel at 50, 65, 85, 105, and 120 days after sowing (DAS) restricted phenological progression and reduced berry weight by 61% as compared to the control at 160 DAS. 500 mg l⁻¹ succinic acid foliar application resulted in maximum plant height (56.4 cm), maximum dry stem weight (DWS) and dry root weight (DRW) whereas 500 mg l⁻¹ ethrel had resulted in minimum plant height and DRW at 180 DAS. During last 50 days of crop growth, the accumulation pattern drastically changed with more than 60% of the biomass allotment to the reproductive part, the berries. The WD in roots ranged between 0.325 mg g⁻¹ and 0.342 mg g⁻¹ during all growth stages. WA content decreased with increase in progression of crop growth and reached the lowest at 180–190 DAS. In a pot experiment, ethrel application up regulated DWF-5 by 2.44, SQE by 3.79 and CYP450s by 1.17 log₂fold in roots 8 h after treatment and succinic acid had up regulated the expression of all these genes by nearly 3 log₂fold change. This is in accordance with the withanolide accumulation pattern in field condition under foliar application of these molecules.

1. Introduction

Ashwagandha (*Withania somnifera* Dunal. Linn.), the Indian ginseng is an important commercial medicinal crop cultivated to harvest mainly roots for their medicinal values. As per ayurveda, ashwagandha roots fall under rasayana category. The literal meaning of rasayana is obtaining the optimum nourishment to the dhatu, the fundamental tissues in human body (National Health Portal Government of India, 2019; <http://www.nhp.gov.in/>). Rasayanas are known to promote health and longevity through enhanced defence against disease, slowing down the process of ageing, revitalising the body in debilitated conditions. In Ayurvedic practice, it is used to calm the mind, relieve weakness and nervous exhaustion. It is also used to promote sexual energy and warrant healthy sleep (Narinderpal et al., 2013). The pharmacological effects of the roots of this plant are attributed to the presence of withanolides, a group of steroidal lactones (Budhiraja and Sudhir, 1987). Leaves of this plants are

used in Ayurvedic and Unani systems for treatment of tumors and tubercular glands (Chopra, 1994). For neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases, the use of ashwagandha was found to be promising (Singh et al., 2011). Withaferin-A (WA), 12-deoxywithastramonolide (WO) and withanolide A (WD) are the major bioactive compounds found in different plant parts of *W. somnifera*. This herb is a part of more than 100 formulations for health perspectives and therefore, there is a huge pharmaceutical demand making this crop a potential crop with significant industrial supplies.

The crop *Withania somnifera* passes through various growth stages. The phenological progression leads to morphological and quality changes in roots (Dhar et al., 2016). The normal vegetative growth of 60 days is required for flower bud initiation in *W. somnifera*. Therefore, changes in phenological progression may alter biomass partitioning as well as the quality parameters in *W. somnifera*. Paclobutrazol (PBZ) had reported to limit production of gibberellin which is required for cell

* Corresponding author.

E-mail address: kuldeep.Kalariya@icar.gov.in (K.A. Kalariya).

elongation and growth in plants. There are reports in which ethylene had inhibited vegetative growth in many plants. Increased content of the immediate precursors of the ethylene and decrease in plant growth is well correlated. Succinic acid is a key intermediate in a primary metabolic pathway leading production of chemical energy in the presence of O₂. Application of PBZ both as a soil drench and foliar application were effective in growth regulation in various crops (Kishore et al., 2015). In India, PBZ and ethephon have been registered as a plant growth regulator under section 9 (3) of Insecticides Act, 1968 in November 2009 by Central Insecticides Board & Registration and is available in the market with various trade names.

The biomass allocation which is the relative amount of biological yield of different plant parts like leaves, stem, roots and reproductive parts is not fixed. The biomass allocation may vary due to crop growth stage, environment condition and species differentiation (Reich, 2002). In ashwagandha, the later growth phase is very much important to get higher biomass production.

Thus, a field experiment was conducted with an objective to study the impact of foliar application of ethrel, PBZ and succinic acid on growth and quality parameters in *W. somnifera*. To study the impact foliar application of ethrel, PBZ and succinic acid at gene expression level, minimum impact of environmental condition is required. Hence, a separate pot experiment under control condition was conducted. Salicylic acid (SA) and methyl jasmonate (JA) are most widely used elicitor molecules in gene expression studies and therefore, they were also included in addition to ethrel, PBZ and succinic acid to study the gene expression pattern.

2. Materials and methods

2.1. Field experiment

A field experiment was conducted during two consecutive winter to spring seasons in year 2016 and 2017 (October 2016–April 2017 and October 2017–April 2018). The experimental location is geographically located on N 22° 55', E 072° 66', MSL – 45 m at the Directorate of Medicinal and Aromatic Plants Research (DMAPR), Boriavi, Anand, Gujarat, India. The average rainfall, maximum and minimum temperature are 800 mm, 42 °C and 12.7 °C, respectively. The water holding capacity and the pH of the experimental sandy loam soil was 20–22% and the 7–7.5, respectively. Total eight treatments were repeated in four replications in randomized block design. Authentic seeds of *W. somnifera* Dunal cultivar JA 134 were procured from Mandor centre of All India Co-ordinated Research Project on Medicinal and Aromatic Plants (AICRP on MAP), Vijaya Raje Scindia, Krishi Vishwa Vidhyalaya, Mandor, Madhya Pradesh, India. Healthy seeds were sown in lines at 30 cm distance. To ascertain maximum germination and full plant strength, the first irrigation was given immediately after sowing. All agronomic practices including weeding, inter culturing, irrigation, fertilizer application etc., were followed as per recommended package of practices of this crop. Among the different treatments, there were no foliar application in T₁: control whereas in T₂: water, T₃: 200 mg l⁻¹ paclobutrazol (PBZ), T₄: 500 mg l⁻¹ PBZ, T₅: 200 mg l⁻¹ ethrel, T₆: 500 mg l⁻¹ ethrel, T₇: 200 mg l⁻¹ succinic acid and T₈: 500 mg l⁻¹ succinic acid was foliar applied at vegetative phase 50 and 65 days after sowing (DAS) and initial reproductive phase (85, 105, and 120 DAS). These growth regulators/retardants were foliar applied during morning hours in such a way to cover different crop growth stage. The spray periods were selected to cover different growth phases i.e. the first spray during vegetative growth period, second and third spray during flowering and berry initiation, respectively, fourth and the fifth spray during berry development phase. Observations on various growth parameters were recorded at four different growth periods to cover different growth stages i.e., stage I (110–115 DAS- berry development), stage II (125–130 DAS- end of berry development to initiation of berry maturity, green berry), stage III (155–160

DAS- berry maturity, green to red berry) and stage IV (180–190 DAS- harvest maturity with brown calyx).

2.2. Measurement of growth parameters

Plant height and root length were measured from freshly harvested plants. Sampled plants were separated into leaves, stem, roots and berries and dried under shade condition. Total arial biomass (TABM) and total biomass (TBM) were calculated based on the leaf dry weight (DWL), stem dry weight (DWS), root dry weight (DWR) and berry dry weight (BDW) measured after proper drying until constant moisture content. Based on the data, the biomass fraction of different organs like leaf mass fraction (LMF), shoot mass fraction (SMF), root mass fraction (RMF) and berry mass fraction (BMF) were calculated. Properly dried plant samples were ground to fine powder for chemical analysis.

2.3. Sample preparation for major withanoloides extraction

One-gram oven dried powdered samples were extracted with methanol (3 × 75 mL, 60 min), under reflux, on water bath maintained at 90 °C. The extracts were filtered, combined and then evaporated to dryness by rotary evaporator under reduced pressure. The dried extract samples were thereafter dissolved in 25 mL methanol and filtered through a 0.45-µm nylon membrane filter (M/s Whatman, India). The 10 µl filtrate samples were injected in UHPLC for analysis.

2.4. UHPLC condition

Ultimate 3000 UHPLC (M/s Thermo Fisher scientific) used for identification and quantification of withanoloides viz withanoloides-A, 12-deoxy withastramonolide and withaferin A. The reference standards were purchased from ChromaDex (CA, USA). The chromatographic separations were carried out by using RP- C18 column (Lichrocart, Merck, 250 × 4.6 mm, 5 µm). Mobile phase, flow rate, column temperature and peaks identification and content quantification were as per method given by Joshi et al. (2010).

2.5. Pesticide residual analysis

2.5.1. Chemicals and reagents

Methanol, water and acetonitrile (MS- grade) were procured from Merck (Darmstadt, Germany) and Formic Acid (99.5+%, Optima™) LC/MS Grade was purchased from Fisher Chemical (Fair lawn, NJ, USA). Paclobutrazol with purity 99.9 % was purchased from Sigma Aldrich (Germany). Stock solution of paclobutrazol (238.72 µg/ml) prepared in methanol and stored at -20.0 °C. The fresh working standards prepared in the range of 1–500 ng/mL from intermediate standard by suitably diluting with MeOH-Water (4:1:v/v).

2.5.2. Sample preparation and extraction procedure

Paclobutrazol residues from ashwagandha roots were prepared as per the method described by Mastovska et al. (2010). In final step, 1 ml of supernatant transferred and evaporated near to dryness and reconstituted with 2 ml of the methanol: water (80:20) for LC-MS/MS analysis.

2.5.3. UHPLC-MS/MS detection

The quantitative analysis of paclobutrazol was performed on Thermo Scientific made TSQ Quantum Access MAX equipped with Dionex made ultra-high performance liquid chromatograph (UHPLC) system (model: Dionex Ultimate 3000 RS). The separation was achieved on Hypersil Gold C18 column (150 × 4.6 mm, 5 µm particle size) with flow rate 0.3 ml/min and column oven temperature 30 °C. An elution gradient was used with solvents A (water with 5mM Ammonium format, 0.1% formic acid) and B (acetonitrile): 0–0.5 min, 98% B; 0.5–2 min, 98-60% B; 2–5 min, 60 -2 % B. The MS parameters of paclobutrazol were optimized in positive ionization mode with capillary voltage 4500 V; vaporizer

temperature 350 °C; sheath gas (N₂) 48 unit; aux gas (N₂) 18 unit and capillary temperature 325 °C. The precursor ion of paclobutrazol was m/z 294.19, and production ion was m/z 125.0, 70.32 for paclobutrazol. The collision energy for product ion m/z 124.99 was 36 eV and for m/z 70.32 was 22 eV. Tube lens value was 145 V for both the product ions.

2.6. Data analysis and presentation

The data were subjected to analysis of variance for the randomized block design. The DSAASTAT Onofri (2007) in MS Excel based add-in module was used for statistical analysis and the least significant differences were calculated to assess the significance of treatment means where the “F” test was found significant at $P < 0.05$.

2.7. Gene expression study

2.7.1. Pot experiment

A pot trial was conducted under control condition for q-PCR gene expression analysis of genes of withanoloid biosynthesis pathway. One healthy plant of ashwagandha was maintained per pot under poly carbonate house. The foliar chemical treatments were given to seedlings as (i) 500 μmethyl jasmonate (MJA) (ii) 500 μM salicylic acid (SA) (iii) 3460 μM ethrel (iv) 1700 μM paclobutrazol (PBZ) (v) 4235 μM succinic acid and distilled water spray as control treatment. The leaves were collected after 8 h of treatments and snap chilled in liquid nitrogen for RNA extraction.

2.7.2. RT-qPCR gene expression analysis of withanoloid biosynthesis pathway

Quantitative real-time PCR was conducted to evaluate the expression levels of sterol Δ7 reductase (DWF-5), squalene epoxidase (SQE) and cytochrome P450 reductase (CYP450s) genes in roots of treated

ashwagandha plants. Total RNA was isolated using total RNA purification kit (Sigma-Aldrich) from roots. The primers were designed from sequences available at NCBI database using primer3 and the quality of primers were checked using oligo analyzer. Total RNA isolation, cDNA synthesis and RT-qPCR conditions and calculation of Log2fold changes are as per described by Kalariya et al. 2019.

3. Results

3.1. Plant growth characteristics

The climatic conditions during both crop period (CP I and CP II) are given in Figure 1 and the field view of the experiment is depicted in Figure 2. The visual observation of different treatments during both the crop periods showed that the plants sprayed with paclobutrazol had leathery dark green leaves whereas plants sprayed with 500 mg l⁻¹ ethrel became stunted with close canopy architecture having arrested berry formation (Figure 3) (till stage III) limiting the berry dry weight by 61% lesser than the control. Plants sprayed with 500 mg l⁻¹ succinic acid were vigorous in growth. The analysis of variance (ANOVA) and least significance difference (LSD) at $P = 0.05\%$ for different parameters studied are presented in Table 1.

Plant height steadily increased from 23.9cm to 50.5 cm from stage I to stage IV. Plant height was minimum in T₆ treated plants across different growth stages (Figure 4 a). Root length increased from 9.4 cm–14.1 cm from stage I to stage IV. The root length became steady after growth stage III (Figure 4b). Mean leaf dry weight was maximum at stage III. Minimum leaf dry weight was noted in T₆ at all growth stages (Figure 4c). Stem dry weight continuously increased from stage I to stage IV. The stem dry weight was maximum in T₈ at stage IV (Figure 4d). Root dry weight and berry dry weight continuously increase with increase in growth stage. Root dry weight was maximum in T₈ and minimum in T₆ (Figure 4e). The

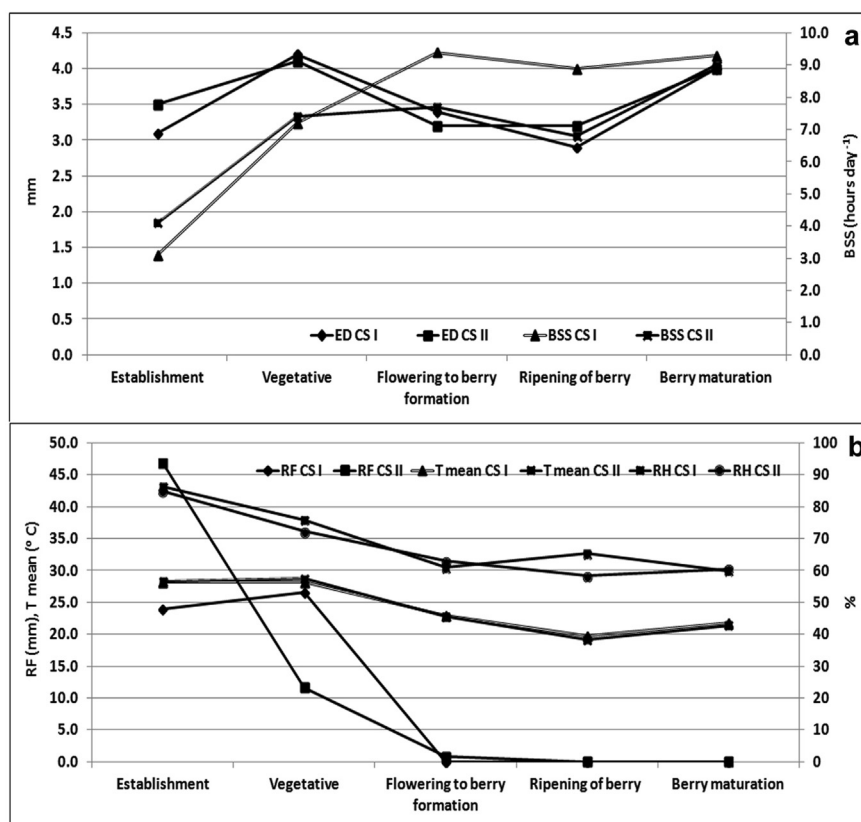


Figure 1. Climatic conditions during crop growth period. (a) evaporative demand (ED) in mm and bright sun shine hours (BSS) (b) rainfall in mm, mean temperature in °C and relative humidity (RH) in % during crop season (CS) I and II.



Figure 2. Ashwagandha plants at 75 DAS. (a) field view of control plants (b) close-up view of control plants (c) field view of plants sprayed with 500 mg l⁻¹ PBZ (d) close-up view of plants sprayed with 500 mg l⁻¹ PBZ (e) field view of plants sprayed with 500 mg l⁻¹ ethrel (f) close-up view of plants sprayed with 500 mg l⁻¹ ethrel (g) field view of plants sprayed with 500 mg l⁻¹ succinic acid (h) close-up view of plants sprayed with 500 mg l⁻¹ succinic acid.

berry dry weight was 0.9 g plant⁻¹ at stage I and reached as high as 38.1 g plant⁻¹ at stage IV (Figure 4f). Treatment T₆ restricted the increase in berry weight leading to only 10.0 g plant⁻¹ as compared to the maximum berry weight of 28.0 g plant⁻¹ in T₄ at stage III.

3.2. Biomass partitioning

The TABM and TBM were 7.9 and 9.0 g plant⁻¹ respectively, at stage I and increased to 61.6 and 65.3 g plant⁻¹ respectively at stage IV (Figure 5 a,b). The organ specific biomass accumulation pattern

changed with progression of growth stage. The LMF which was 0.364 at stage I decreased continuously and reached as low as to 0.063 at stage IV. The LMF was maximum in T₆ at stage I, II and III (Figure 5 c). The SMF was 0.416 at stage I and stage II and decreased to 0.301 at stage IV. The SMF was maximum in T₆ at stage II and III (Figure 5 d). The RMF was decreased continuously across the stages. It was 0.122 at stage I and decreased to 0.058 at stage IV (Figure 5 e). The berry mass fraction was 0.097 at stage I and reached to the maximum of 0.578 at stage IV. It is interesting to note that the stem was active sink till stage II whereas stage III onwards the active sink was berry which resulted in

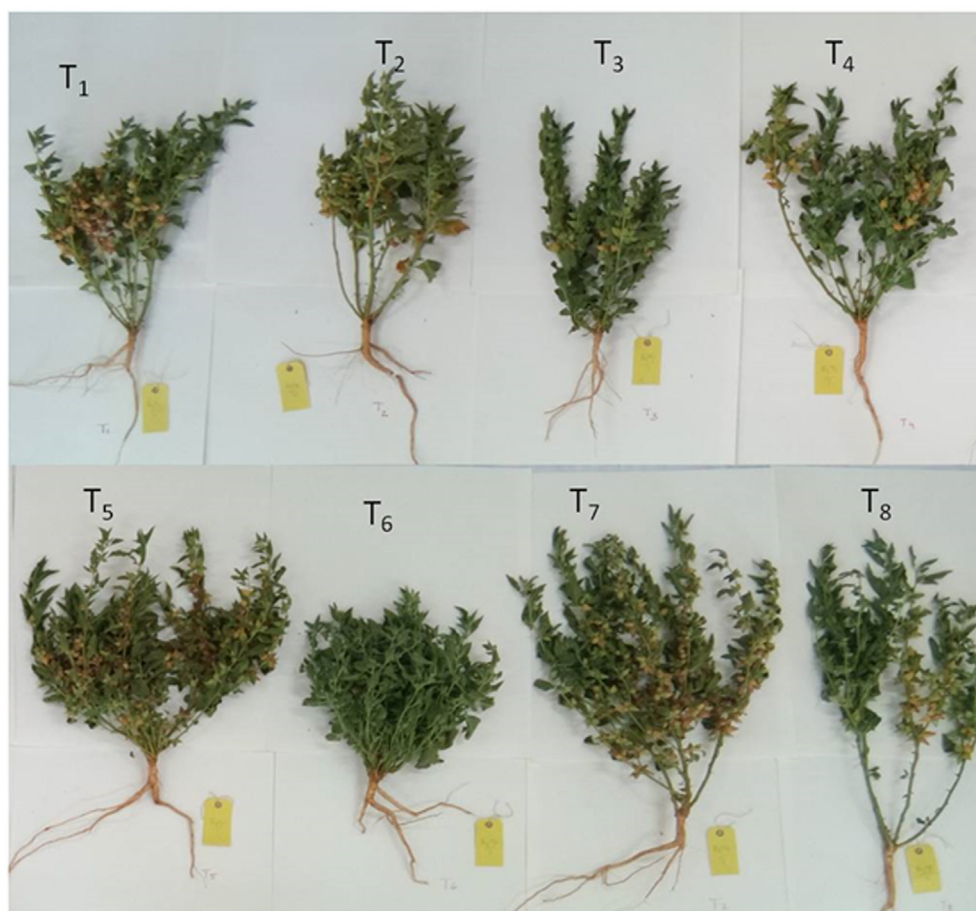


Figure 3. Growth behaviour of ashwagandha at 155 DAS under different treatment. T₁: control T₂: water, T₃: 200 mg l⁻¹ paclobutrazol (PBZ), T₄: 500 mg l⁻¹ PBZ, T₅: 200 mg l⁻¹ ethrel, T₆: 500 mg l⁻¹ ethrel, T₇: 200 mg l⁻¹ succinic acid and T₈: 500 mg l⁻¹ succinic acid foliar applied at 50, 65, 85, 105, and 120 DAS.

Table 1. Analysis of variance (ANOVA) and least significance difference (LSD) at P = 0.05% for different parameters studied.

EFFECT	DF	PLH	RTL	DWL	DWS	DWR	DWB	TABM	TBM	LMF	SMF	RMF	BMF
Stage	3	4.89	1.91	1.02	1.54	0.36	2.32	4.15	4.40	0.018	0.019	0.008	0.026
Year X Stage	3	NS	2.70	1.44	2.18	NS	3.27	5.86	NS	0.025	0.027	NS	0.037
Treatment	7	2.71	NS	0.76	1.86	0.32	3.69	5.46	5.62	0.017	0.021	0.011	0.027
Year X Treatment	7	NS	NS	1.07	2.63	NS	NS	NS	NS	0.024	0.029	NS	0.038
Stage X Treatment	21	NS	NS	1.52	NS	NS	NS	NS	NS	0.034	0.042	0.021	0.054
Year X Stage X Treatment	21	NS	NS	2.15	5.25	NS	NS	NS	NS	NS	0.059	0.030	0.076

PLH-Plant height, RTL-root length DWS-dry shoot weight, DWR-dry root weight, DWL-dry leaf weight, DWB-dry berry weight, TABM-total aerial biomass, TBM-total biomass, LMF-leaf mass fraction, SMF-shoot mass biomass, RFM-root mass biomass, BMF- berry mass fraction.

as high as nearly 60% of the total biomass allocation in berry in just 80 days period (Figure 5 f). The BMF was less than 37% at stage III in T₆ treated plants.

3.3. Major withanoloides content in roots at different growth stages

The HPLC chromatogram of major withanoloides are given in Figure 6. Two years mean WA content in roots decreased with increase in progression of crop growth and reached the lowest at stage IV. A very slow rate of increase in WO in roots is noted till stage III which then reached to the maximum at stage IV. Mean WA in roots was 0.483 mg g⁻¹ at stage I, 0.448 mg g⁻¹ at stage II and decreased to 0.338 mg g⁻¹ stage III and further drastically decreased to 0.184 mg g⁻¹ at stage IV accounting 62% reduction as compared to the stage III. Among all the treatments, T₃ had the highest WA in roots at stage I whereas it was maximum in T₇ at

stage IV. The highest WO in roots was in T₈ (0.180 mg g⁻¹) followed by T₇ (0.170 mg g⁻¹) at stage IV. The mean WD in roots ranged between 0.325 mg g⁻¹ and 0.342 mg g⁻¹ during all growth stages. There was a mix response in WD content with growth stage except in T₈ in which the WD continuously increased from 0.336 mg g⁻¹ stage I to 0.397 mg g⁻¹ stage IV (Figure 7).

3.4. Pesticide residue

The chromatograph of paclobutrazol showing paclobutrazol standard and sample is given in Figure 8. The residues of paclobutrazol in ashwagandha roots were below quantification limit (BQL) i. e. 0.006 mg/kg at stage I. During second and third stage the residues of paclobutrazol were in the range of 0.06–0.09 mg/kg at either dose (Table 2). Subsequently, the concentration of paclobutrazol slowly and steadily declined

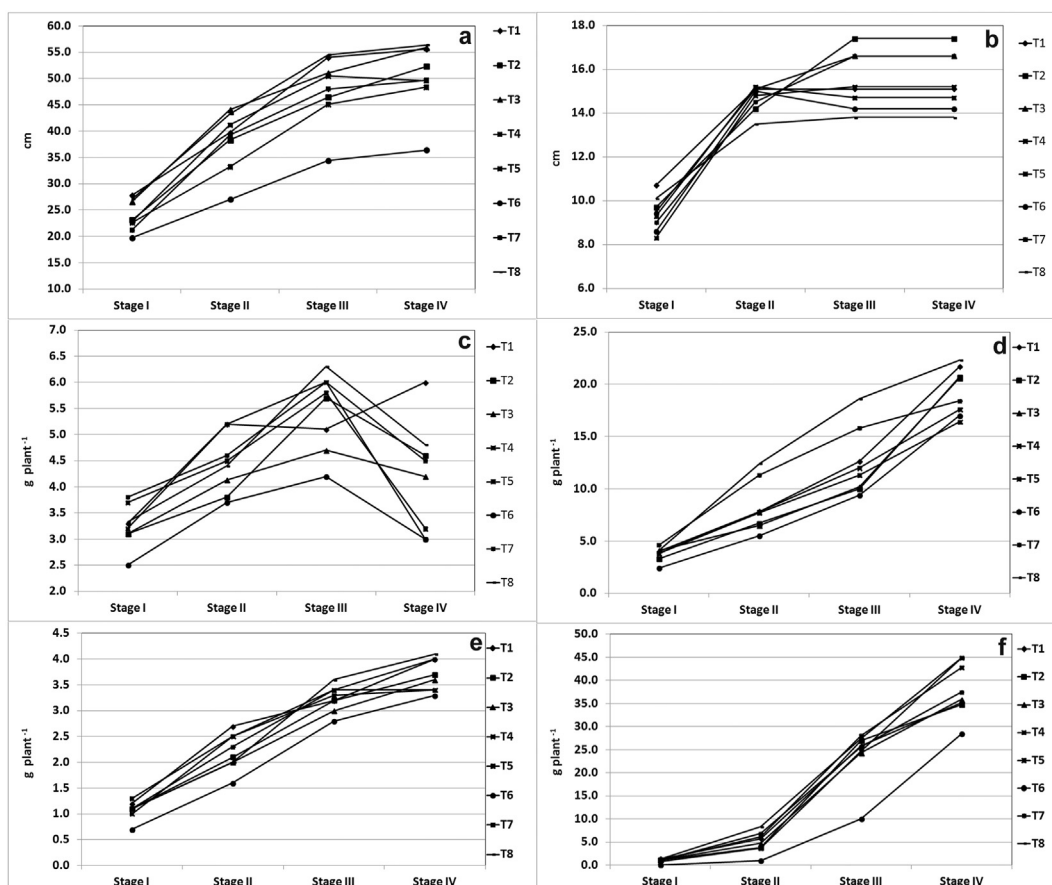


Figure 4. Growth parameters in *W. Somnifera* during different growth stages. (a) plant height (b) root length (c) dry leaf weight (d) dry stem weight (e) dry root weight (f) dry berry weight.

with the time, which has been reflected from the BQL values observed at stage-IV on both doses. However, the samples from remaining treatments were free from paclobutrazol residues.

3.5. Differential expression of genes involved in withanoloides biosynthesis in response to foliar application

The primer sequences used to monitor expression profiling of different genes on real time basis are mentioned in Table 3. The expression levels in terms of log2fold changes of sterol $\Delta 7$ reductase (DWF-5), squalene epoxidase (SQE) and cytochrome P450 reductase (CYP450s) conferring increase in withanoloides contents in roots varied under different treatments. The result showed that the expression of SQE, CYP450s and DWF-5 genes in roots increases due to application of all tested elicitors except CYP450 under PBZ application and DWF-5 under SA application after 8 h of foliar application (Figure 9). Ethrel application increased expression of DWF-5 by 2.44 log2fold, SQE by 3.79 log2fold and CYP450s by 1.17 log2fold in roots 8 h after treatment. The MJA had maximum positive impact with more than 8 log2fold change during expression profiling of these genes on real time basis. Succinic acid had also positively up regulated the expression of these by nearly 3 log2fold changes.

4. Discussion

PBZ application prevents synthesis of Gibberellins (GAs) by its interaction with kaurene oxidase, a cytochrome P-450 oxidase, and inhibits the microsomal oxidation of kaurene, kaurenal, and kaurenol.

Decrease cell division and elongation at the apical meristem of the shoot are results of reduced GAs but, has little effect on the production of leaves or root growth (Lambers et al., 1995). In our study, the reduction in dry weight per plant under 500 mg l^{-1} PBZ applications seems to be the results of antagonistic effects of GAs. The ethylene had been reported to act as both stimulator as well as an inhibitor in plants but, largely was as a growth inhibitor at later growth stage and as growth promoter at early growth stage Sharp and Le Noble (2002). The PBZ treated plants senesced slowly compared to the control plants. The delayed onset of senescence in withania leaves seems probably by enhancement effects of PBZ on endogenous levels of cytokinins promoting chlorophyll formation and increased activity of certain antioxidant enzyme (Synkova et al., 2006).

High SMF and LMF in T_6 during stage I to III was because the ethrel application had restricted berry growth and hence, the photosynthates was incorporated in active sinks like leaf and stem. Decrease in RMF and increase in BMF towards maturity progression showed that berry became the active sinks.

Ethrel, after penetration into the plant tissues, is translocated and progressively decomposed to ethylene which reduces apical dominance and thereby increases axillary branching. The ethylene causes opposite effects of gibberellins in plants and thus changes the source-sink relationship. The delayed berry formation was because of the ethylene had led to the arrested vegetative growth. Prior to flowering, application of ethylene can result in flower abortion and delayed flowering.

Succinate, a key intermediate in the tricarboxylic acid cycle, is considered as the mimetic of salicylic acid for imparting resistance to abiotic stresses in plants (Gupta et al., 2017). Exogenous application of

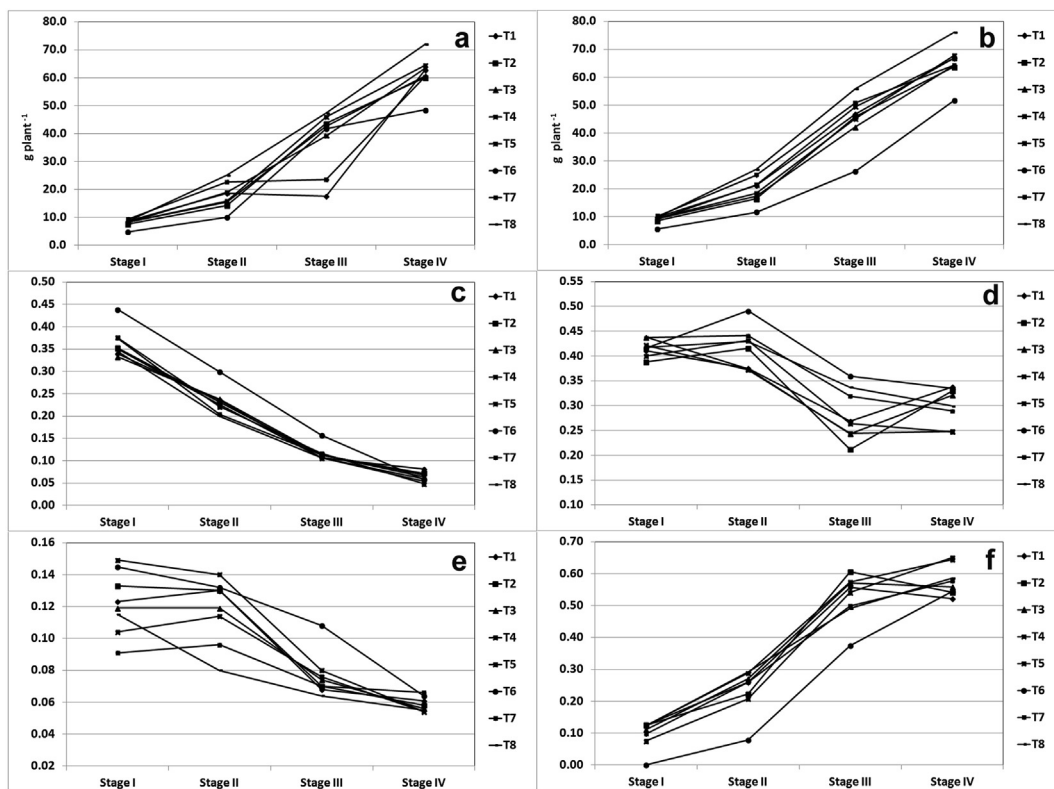


Figure 5. Biomass partitioning in *W. Somnifera* during different growth stages. (a) total aerial biomass (b) aerial biomass (c) leaf mass fraction (d) stem mass fraction (e) root mass fraction (f) berry mass fraction.

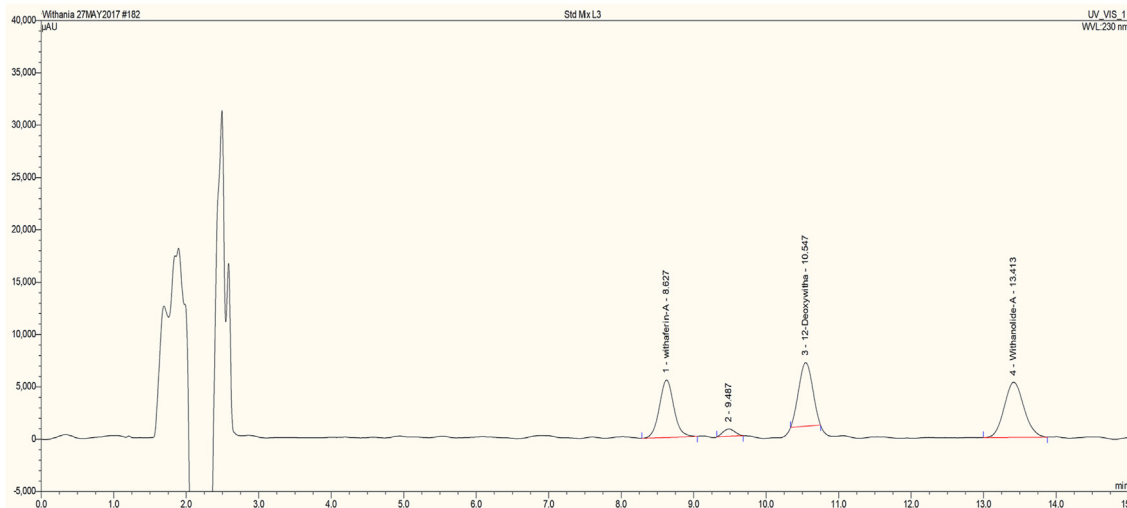


Figure 6. HPLC chromatogram of major withanoloides standards.

succinic acid had beneficial impacts on mineral nutrient uptake and caused increase in biomass in alfalfa plants under aluminium toxicity (An et al., 2014). Foliar application of succinic acid successively at different growth stages would have facilitated biomass partitioning along with quality partitioning in terms of high WD accumulation in roots.

Paclobutrazol was reportedly absorbed through the roots and transported primarily through stem (via xylem) before accumulating in the leaves (Wang et al., 1986). Thus, the plants roots are the main avenue of PBZ absorption storage and distribution. The results obtained in the study reveals that the residues of paclobutrazol were BQL i.e. 0.06 mg/kg from the root samples of ashwagandha collected at first stage which was just

after the second spray of paclobutrazol applied at the rate of 200 and 500 mg/l-1. It is worth to mention that the PBZ application had not any beneficial effects on root weight or withanolides content. The residues of paclobutrazol recorded on first stage were on expected line because paclobutrazol is believed to be readily absorbed by plants through roots but due to foliar spray its residues were not detected in roots during the first stage. However, on later stage root system of ashwagandha had absorbed the paclobutrazol residues deposited on soil due to foliar spray losses. This might be a probable reason of detection of paclobutrazol residues on II&III growth stage of ashwagandha. The rate of loss of paclobutrazol residue dissipation is quite slow as its residues detected on II

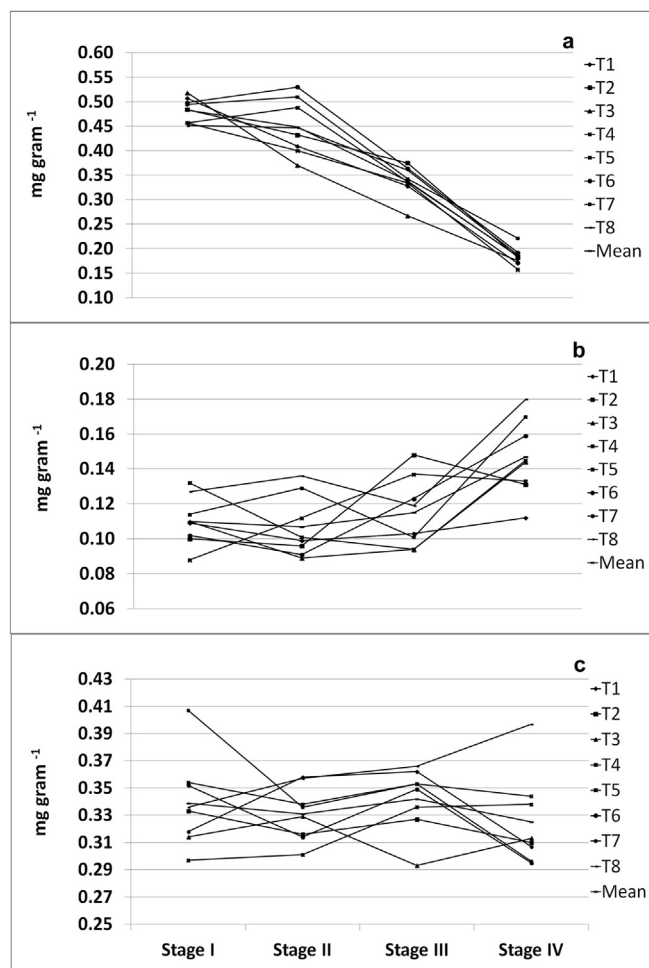


Figure 7. Content and accumulation pattern of (a) WA, (b) WO and (c) WD in roots of *W. somnifera* at different growth stages. (a).

&III growth stage of ashwagandha were quantitatively more or less similar but were BQL (<0.006 mg/l) on stage IV. It was reported that paclobutrazol residues in roots of *Nerium oleander* L. seedlings remained constant during the experimental period. The high level of PBZ residues detected in the substrate 30 days after application had decreased significantly (by 67%) by the end of the experiment (Ochoa Rego et al., 2009).

According to article 12 of Regulation (EC) No 396/2005, the European Food Safety Authority (EFSA) has reviewed the maximum residue levels (MRLs) for the pesticide active substance paclobutrazol by assessing the occurrence of paclobutrazol residues in plants, processed

commodities, rotational crops and livestock. Based on the assessment of the available residue data and a consumer risk assessment, no apparent risk to consumers due to paclobutrazol was identified and established (Brancato et al., 2017) Although, the MRL value of the paclobutrazol is not available for Ashwagandha (*Withania somnifera* Dunal. Linn.) but the EU (EU, 2019) has suggested a default value of 0.02 mg/kg for roots of under the section teas, coffee, herbal infusions, cocoa and carobs (http://ec.europa.eu/food/plant/pesticides/eu-pesticides_database/public). Consumption of ashwagandha could be safe although the residues of paclobutrazol were detected on II & III stage were above MRL value 0.02 mg/kg as farmers are predominantly harvest the ashwagandha crop after 4th stage which lies after 180 DAS when the residues of paclobutrazol were BQL (<0.006 mg/kg). Further, as a farmers practice, the harvested roots of ashwagandha are subjected to partial shade for proper drying could provide ample time where the concentration of trace amount of paclobutrazol could be reduced further.

Withanoloides are synthesized through sterol pathway the first step of which is conversion of squalene into 2,3 Oxidosqualene under the action of SQE gene. The conversion of 5-dehydroavenosterol to Δ5-avenosterol/isofucosterol is carried out by DWF 5. Cyclization of 24-methylene cholesterol compounds into withanoloides is mediated by CYP450s catalyzed (Gupta et al., 2013). In the current pot study, we found increased expression of important enzymes involved in withanoloides biosynthesis pathway under elicitor molecules i.e. SA and JA as well as ethrel and succinic acid application. This increased regulation of DWF-5 and SQE in roots up to four-fold after 8 h after treatment of foliar ethrel and SQE and CYP450s gene under succinic acid application after 8 h of treatment established their role in withanaloide biosynthesis pathway.

In green plants, photosynthesis is the sole source of energy and thus a plant has a limited amount of energy to divide between the multiple functions at a time. Plants have an option to put this energy in different proportion into different plant parts besides fulfilling the energy required for maintenance, storage and defence mechanism. Plants can decide priority in partitioning of this energy among various organs. In a medicinal plant the yield of an active principal compound depends on the content of the compound per unit weight of different plant parts. Thus, the quantitative understanding of functional partitioning is more complex in a medicinal plant than other crops. The rate of structural growth, storage, respiration and exudation of a root together decide the phloem import capacity to transport photosynthates from leaf to the roots. But reports are there in which roots have been shown as a poor sink (Brouwer, 1983). Reports are there in which exogenous application of succinic acid had positive impacts on mineral nutrient uptake and thereby increased biological yield in plants (An et al., 2014).

Organ specific biomass accumulation pattern changed with progression of growth stage in ashwagandha. It is interesting to note that the stem was active sink till stage II whereas stage III onwards the active sink was berry which resulted in as high as nearly 60% of the total biomass allocation in berry in last 80 days crop period. 500 mg l⁻¹ can be used to

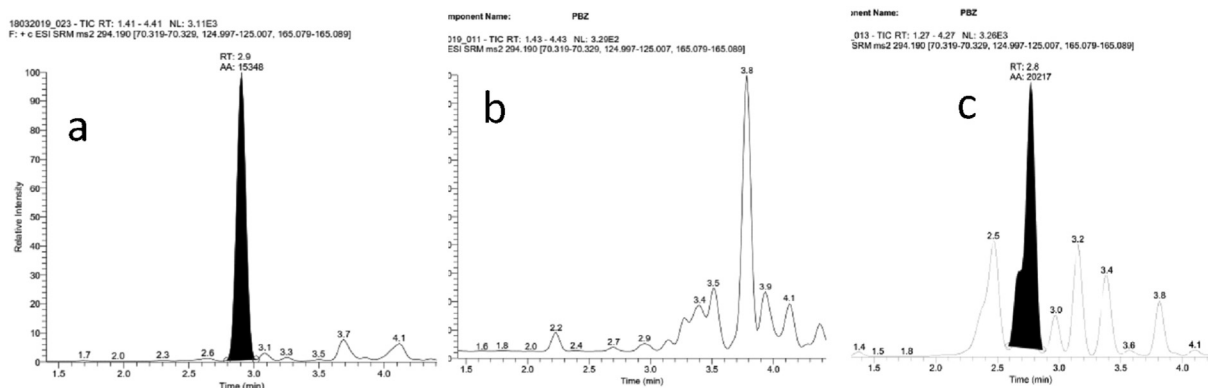


Figure 8. Chromatograph of paclobutrazol showing (a) Paclobutrazol standard 50 ppb (b) sample blank (c) sample.

Table 2. Residue of paclobutrazol in the roots of Ashwagandha.

Treatment Detail	Stage-I 110-115 DAS	Stage-II 125-130 DAS	Stage-III 155-160 DAS	Stage-IV 180-190 DAS
T ₁	BQL#	BQL	BQL	BQL
T ₂	BQL	BQL	BQL	BQL
T ₃	BQL	0.06ppm	0.05ppm	BQL
T ₄	BQL	0.09ppm	0.08ppm	BQL
T ₅	BQL	BQL	BQL	BQL
T ₆	BQL	BQL	BQL	BQL
T ₇	BQL	BQL	BQL	BQL
T ₈	BQL	BQL	BQL	BQL

Stage-I	Stage-II	Stage-III	Stage-IV
had 04 sprays at 50,65,85,105 DAS	had 05 sprays at 50,65,85,105 and 120 DAS	had 05 sprays at 50,65,85,105 and 120 DAS	had 05 sprays at 50,65,85,105 and 120 DAS
Samplings at 110–115 days	Samplings at 125–130 days	Samplings at 155–160 days	Samplings at 180–190 days
Sampling time from last spray-05 days	Sampling time from last spray-05 days	Sampling time from last spray-35 days	Sampling time from last spray-60 days

BQL=below quantification limit, DAS- days after sowing.
 # BQL=<LOQ i.e. 0.006 mg/kg.

Table 3. qRT-PCR primer sequences for gene expression analysis.

Gene	Sequences	Tm	GC content (%)
CYP450s	CCTGCTGACCCCTCAACTCC	60.32	60
	CITTCGTGGCCCTCCCTT	59.89	55
DWF-5	CITTAGCTCGCACTTCCACT	59.75	55
	GCATCGGTGTCATCCCTTT	60.46	55
SQE	GCTACAACCACCACTACCACTACA	57.38	50
	GTAGCCACCAGGTTGTAGGAGTTC	59.09	54

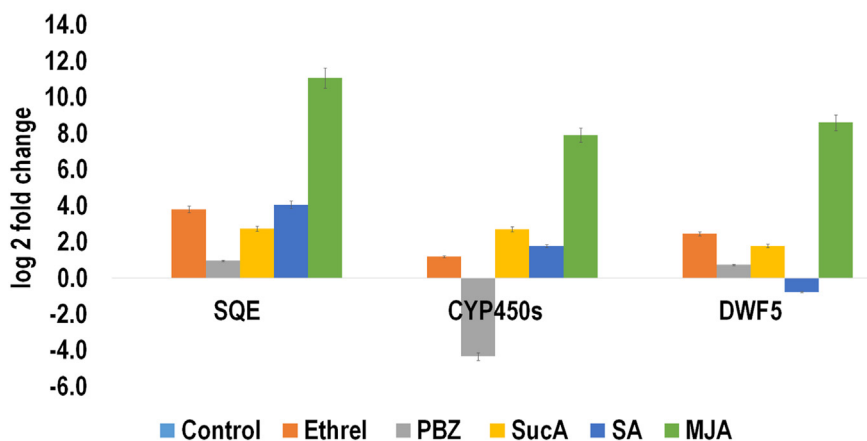


Figure 9. Log2fold changes in expression of DWF-5, SQE and CYP450s in roots of *W. somnifera*.

delay berry formation and 500 mg l⁻¹ succinic acid foliar application can be used to harvest greater biomass including root yield along with higher WO in roots. The PBZ application had no beneficial effects on root weight or withanolides content however, if applied as fungicide the residues of paclobutrazol in ashwagandha roots remains below the quantification limit 70 days after foliar application. Over expression of DWF-5, SQE and CYP450s in roots 8 h after treatment of ethrel and succinic acid are in conformity with the improved quality of ashwagandha in field condition due to foliar application of these molecules.

Declarations

Author contribution statement

Kuldeepsingh A. Kalariya: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Narendra A. Gajbhiye: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Dipal Minipara, Sushil Kumar, Vanrajsinh Solanki, Susheel Singh: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Parmeshwar L. Saran: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Prince Choyal: Contributed reagents, materials, analysis tools or data.

Ponnuchamy Manivel: Analyzed and interpreted the data.

Funding statement

This work was supported by Indian Council of Agricultural Research, New Delhi.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We are grateful to the Director, ICAR-Directorate of Medicinal and Aromatic Plants Research, Boriavi, Gujarat for providing all basic facilities. We are also thankful to AICRP on MAP, Mandsoor, Centre, VRSKVV, Madhya Pradesh, India for providing seed material used in this study.

References

- An, Y., Zhou, P., Xiao, Q., Shi, D., 2014. Effects of foliar application of organic acids on alleviation of aluminum toxicity in alfalfa. *J. Plant Nutr. Soil Sci.* 177 (3), 421–430.
- Brancato, A., Brocca, D., De Lentdecker, C., Erdos, Z., Ferreira, L., Greco, L., Janossy, J., Jarrah, S., Kardassi, D., Leuschner, R., 2017. European Food Safety Authority (EFSA). Review of the existing maximum residue levels for paclobutrazol according to Article 12 of Regulation (EC) No 396/2005. *EFSA Journal* 15 (8), e04974.
- Brouwer, R., 1983. Functional equilibrium: sense or nonsense? *NJAS - Wageningen J. Life Sci.* 31 (4), 335–348.
- Budhiraja, R.D., Sudhir, S., 1987. Review of biological activity of withanolides. *J. Sci. Ind. Res.*
- Chopra, R.N., 1994. *Glossary of Indian Medicinal Plants*. Academic Publishers India, New Delhi.
- Dhar, R.S., Khan, S., Khajuria, R.K., Bedi, Y.S., 2016. Dynamics of squalene content in different tissues of Ashwagandha (*Withania somnifera* L. Dunal) during its growth phases. *Ind. Crop. Prod.* 84, 375–380.
- U, 2019. Pesticide database. Downloaded from <http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=pesticide.residue>. CurrentMRL&language=EN Date :17-07-2019.
- Gupta, P., Goel, R., Pathak, S., Srivastava, A., Singh, S.P., Sangwan, R.S., Asif, M.H., Trivedi, P.K., 2013. De novo assembly, functional annotation and comparative analysis of *Withania somnifera* leaf and root transcriptomes to identify putative genes involved in the withanolides biosynthesis. *PLoS One* 8 (5).
- Gupta, A., Hisano, H., Hojo, Y., Matsuura, T., Ikeda, Y., Mori, I.C., Senthil-Kumar, M., 2017. Global profiling of phytohormone dynamics during combined drought and pathogen stress in *Arabidopsis thaliana* reveals ABA and JA as major regulators. *Sci. Rep.* 7 (1), 1–13.
- Joshi, C., Gajbhiye, N., Phurailatpam, A., Geetha, K.A., Maiti, S., 2010. Comparative morphometric, physiological and chemical studies of wild and cultivated plant types of *Withania somnifera* (Solanaceae). *Curr. Sci.* 644–650.
- Kalariya, Kuldeepsingh, Gajbhiye, N., Minipara, D., Meena, R.P., Kumar, S., Saha, A., Trivedi, A., Manivel, P., 2019. Deep sequencing-based de novo transcriptome analysis reveals biosynthesis of gymnemic acid in *Gymnema sylvestris* (Retz.) SchultD. *Ecol. Genet. Genom.*
- Kishore, K., Singh, H.S., Kurian, R.M., 2015. Paclobutrazol use in perennial fruit crops and its residual effects: a review. *Indian J. Agric. Sci.* 85 (7), 863–872.
- Lambers, H., Nagel, O.W., Van Arendonk, J.J.C.M., 1995. The control of biomass partitioning in plants from favourable and stressful environments: a role for gibberellins and cytokinins. *Bulg. J. Plant Physiol.* 21 (2-3), 24–32.
- Mastovska, K., Dorweiler, K.J., Lehotay, S.J., Wegscheid, J.S., Szpylka, K.A., 2010. Pesticide multiresidue analysis in cereal grains using modified QuEChERS method combined with automated direct sample introduction GC-TOFMS and UPLC-MS/MS techniques. *J. Agric. Food Chem.* 58 (10), 5959–5972.
- Narinderpal, K., Junaid, N., Raman, B., 2013. A review on pharmacological profile of *Withania somnifera* (Ashwagandha). *Res. Rev. J. Bot. Sci.* 2, 6–14.
- National health portal, government of India. https://www.nhp.gov.in/rasayana_mtl2019, 2019–. (Accessed 3 October 2019).
- Ochoa Rego, J., Leemhuis, F., Antonio, J., Bañón Arias, S.D.P., Fernández Hernández, J.A., 2009. Distribution in Plant, Substrate and Leachate of Paclobutrazol Following Application to Containerized Nerium Oleander L. Seedlings.
- Onofri, 2007. *Data Analysis and Presentation*. Chapter: 6 Data Analysis and Interpretation.
- Reich, P.B., 2002. Root-shoot Relations: Optimality in Acclimation and Adaptation or the 'Emperor, S New Clothes. *Plant Roots: the Hidden Half*, pp. 205–220.
- Sharp, R.E., LeNoble, M.E., 2002. ABA, ethylene and the control of shoot and root growth under water stress. *J. Exp. Bot.* 53 (366), 33–37.
- Singh, N., Bhalla, M., de Jager, P., Gilca, M., 2011. An overview on ashwagandha: a rasayana (Rejuvenator) of ayurveda. *Afr. J. Tradit., Complement. Altern. Med.* 8 (5S).
- Synkova, H., Semoradova, S., Schnablová, R., Witters, E., Husak, M., Valcke, R., 2006. Cytokinin-induced activity of antioxidant enzymes in transgenic Pssu-ipt tobacco during plant ontogeny. *Biol. Plantarum* 50 (1), 31–41.
- Wang, S.Y., Sun, T., Faust, M., 1986. Translocation of paclobutrazol, a gibberellin biosynthesis inhibitor, in apple seedlings. *Plant Physiol.* 82 (1), 11–14.