Chapter 2
Influence of Macronutrient Deficiencies on Molybdenum Content in Ashwagandha Roots and its Correlation with Withanolide Content
2.1. Introduction

For complete growth of medicinal plants, it requires 17 elements: carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, iron, manganese, zinc, copper, boron, molybdenum, chlorine and nickel (HamLin, 2006). If nutrients are not available during growth phase of the plants, deficiency symptoms will occur and quality of product will deteriorate. Essential elements used by plants in relatively large amounts for growth are termed as macronutrients. The major macronutrients are nitrogen (N), phosphorous (P), potassium (K) and Calcium (Ca). Molybdenum (Mo) is a micronutrient that governs biosynthesis and transport of secondary metabolites (HamLin, 2006). All these macronutrients (N, P, K, and Ca) deficiencies may influence micronutrient uptake including Mo (a micronutrient). Micronutrients are important for promoting plant growth and influence biosynthesis of secondary metabolites (Brady and Weil, 1999).

Secondary metabolites differ with location, cultivation practices, soil nutrients, tissue type and varieties of Ashwagandha (Kumar et al., 2007; Kubsad et al., 2009; Nagella and Murthy, 2012). It is known that the medicinal properties of Ashwagandha are attributed to withanolides. Ashwagandha roots are used in several medicinal formulations in the form of decoctions, infusions, ointments, powder and syrups. It may be expected that variations in macro- and micronutrients can influence the growth of plants and also biosynthesis/ transport of withanolides (Suryapujari et al., 2010). Such, variations leads to inconsistent product formulations resulting in unreliable therapeutic and health promoting effects (Patwardhan et al., 2009). Hence the objective of the present study was to examine how the contents of withanolides are influenced under macronutrient deficiency conditions.
2.2. Material and Methods

2.2.1. Chemicals
Ferric chloride hexahydrate, orthophosphoric acid, sulphuric acid, methanol, chloroform, nitric acid, hydrochloric acid, 2, 2-diphenyl-1-picryl-hydrazyl, dichloromethane, toluene, acetone, diethyl ether, cholesterol were procured from local supplier.

The standard withanoside V (WS V), withaferine A (WF A), 1, 2 deoxywithastra-monolide (1, 2 DWM), withanone (WNN), withanolide A (WN A) and withanolide B (WN B) were purchased from Natural Remedies Pvt. Ltd. Bangalore India. Pre-coated silica gel plates [0.2mm thickness, 60F$_{254}$ (20 cm X 20 cm)] were obtained from Merck Ltd. Mumbai, India.

2.2.2. Plant Materials
Ashwagandha var. JS-134 was cultivated under Indian Council of Agricultural Research- National Agriculture Innovation Project III (ICAR-NAIP III) in Vidarbha region of Maharashtra, India (Fig 2.1). Based on the macronutrient deficiency symptoms nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K), calcium deficient (-Ca), as well as healthy (control) Ashwagandha fresh plants were identified and collected in the month of November and December 2011 from different areas of Vidarbha (Suryapujari et al., 2010). In each group 15 plant sample were used for investigation.
Fig. 2.1: Cultivation of Ashwagandha var. JS-134 under National Agriculture Innovation Project - Sustainable Rural Livelihood Security in Backward Districts of Maharashtra (NAIP-III).
Chapter 2: Influence of Nutrients Deficiency on Withanolides Content

2.2.3. Determination of Nitrogen, Phosphorus, Potassium and Calcium

Estimate of nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) were carried out as per the method described by Suryapujari et al. (2010).

2.2.4. Determination Molybdenum (Mo)

1 g root samples were subjected to acid digestion with 5 mL of concentrated HNO₃. To the digested sample 2 mL concentrated HCl was added and the samples were diluted to 25 mL with deionized water.

Stock solution 1 g Mo was dissolved in minimum amount of deionized water and HN0₃, and 8 mL HCl was added and was diluted with deionized water to 1L. Working Mo standards 25, 50 and 100 ppm were prepared by diluting the stock solution. Determination of Mo content was carried out by Atomic Absorbance Spectroscopy (AAS) (Hanlon, 1998).

The amount of Mo was estimated by formula:

\[
\text{Mo (ppm)} = \frac{\text{absorbance of sample} \times \text{dilution factor}}{\text{Sample weight}}
\]

2.2.5. Preparation of Root Extracts for Determination of Withanolides Content

As per the method described by Chaurasiya et al. (2008) the fresh root (5.0 g) were finely powdered in liquid nitrogen and extracted overnight in 20 mL of methanol–water (25:75, v/v) at room temperature (25°C) on a shaker and filtered. The filtrate was further extracted with n-hexane (3 × 60 mL). The n-hexane fraction was discarded and the methanol–water fraction was further extracted with 3 times 60 mL chloroform. The chloroform fractions were pooled and concentrated to a dry powder. The residue was weighted and redissolved in chloroform to give 20 mg/mL samples.
2.2.6. Preparation of Standard Withanolide Solutions
Stock solutions (1 mg/mL) were prepared in chloroform. 4–24 µL of working standards (50 ng/mL made in chloroform) were applied to HPTLC plate to obtain six point calibration curves. The chloroform extracts of the roots were subjected to HPTLC analysis for the quantification of the individual withanolides.

2.2.7. Preparation of Colour Reagent for Total Withanolide Determination
8 mL of stock solution (21.5 g ferric chloride hexahydrate dissolved in 100 mL orthophosphoric acid) was diluted to 100 mL with conc. sulphuric acid.

2.2.8. Determination of Total Withanolides (TW) Content
A modified spectrometric method was used to determine total withanolides content in roots. For this purpose 1 mL chloroform extract was used for the development of color by adding 2 mL of glacial acetic acid and 21 mL of color reagent (Mishra, 1994). After keeping for 5 min in an ice bath, the optical density was recorded in a spectrophotometer at 540 nm. The concentration of withanolides was calculated using cholesterol as standard.

2.2.9. Quantification of Major Withanolides (MW)
Quantification was carried out by HPTLC chromatography using dichloromethane: toluene: methanol: acetone: diethyl ether (7.5:7.5:3:1:1 v/v) as the mobile phase. The chromatogram was run for 80 mm. Quantitative evaluation of the plate was performed in the absorption reflection mode at 235 nm, tungsten (W) and deuterium (D2) lamp, using slit width: 4.00 x 0.30 mm, data resolution 100 µm/step, scanning speed: 20 mm/s, and base line correction was used (Sharma et al., 2007)
2.2.10. Estimation of Free Radical Scavenging Activity by 2, 2-diphenyl-1-picryl-hydrazil (DPPH) Method

Free radical scavenging activity of Ashwagandha extract was measured by 2, 2-diphenyl-1-picryl-hydrazil (DPPH) method with some modification (Shimada et al., 1992).

1 mL of 0.1 mM solution of DPPH in methanol was added to 0.5 mL of 100 μg extract of all groups. After 30 min absorbance was measured at 517 nm. The percentage of DPPH inhibition was calculated by using equation:

\[ \text{Inhibition} \, (%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]

Where,
\[ A_0 = \text{Absorbance of the blank} \]
\[ A_1 = \text{Absorbance of 100 μg of chloroform root extract} \]

2.2.11. Statistical analysis

The results are expressed as mean of ± standard deviations. The data were analyzed by standard computer program: Excel (2003) and statistical analysis was carried out by one-way ANOVA test.

2.3. Results

2.3.1. Symptoms of Nitrogen Deficiency

Leaves get yellow and detached earlier. Yellowing starts from tip of leaf to lower portion. Before shedding, young leaves curved and flower and fruit formation delays by 17 days. Nitrogen deficiency leads to reduced shoot and root growth. Plants had dull appearance and excess deficiency led to complete defoliation and death of plant prematurely (Fig. 2.2). Average nitrogen content was reduced 49.8% in deficient sample when compared with healthy sample (Table 2.1).

2.3.2. Symptoms of Phosphorous Deficiency

Leaves became yellow and turned into brown color from centre to other part of leaf. Defoliation of old leaves was noticed earlier than new leaves. Leaves became curved and misshapen. Leaves started
coiling inside due to phosphorous deficiency (Fig. 2.2). Phosphorous content was reduced by 88.0% in deficient sample when compared with healthy sample (Table 2.1).

2.3.3. Symptoms of Potassium Deficiency
Brown spots were observed on potassium deficient leaves. Burning of leaves at tip was observed and plants started drying from shoot to root. Leaves showed brown and yellow patches (Fig. 2.2). Potassium content was reduced by 88.7% in deficient samples when compared with healthy samples (Table 2.1).

2.3.4. Symptoms of Calcium Deficiency
Deficiency of calcium showed rotting of tip of leaf, roots and shoots. Seeds were malformed and cracking of capsules was observed. In case of excess deficiency, complete drying of plant was observed and seeds remained immature (Fig. 2.2). Calcium content was reduced by 38% in deficient samples when compared with healthy sample (Table 2.1).
**Fig. 2.2:** Macronutrient deficiency symptoms in Ashwagandha plant; healthy (control), nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K) and calcium deficient (-Ca) plant.


**Table 2.1:** Concentration of nitrogen, phosphorus, potassium and calcium in the roots of deficient plant as compare to healthy plants. Each value is mean of fifteen independent samples.

<table>
<thead>
<tr>
<th></th>
<th>Concentration of N</th>
<th>Concentration of P</th>
<th>Concentration of K</th>
<th>Concentration of Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>-N</td>
<td>Control</td>
<td>-P</td>
</tr>
<tr>
<td>% Content</td>
<td>1.44</td>
<td>0.94</td>
<td>1.10</td>
<td>0.22</td>
</tr>
<tr>
<td>% Reduction</td>
<td>49.8</td>
<td>88.0</td>
<td>88.74</td>
<td>38.0</td>
</tr>
</tbody>
</table>

healthy (control), nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K) and calcium deficient (-Ca)
2.3.5. Major Withanolide (MW) by HPTLC
A clear-cut separation of major withanolides was achieved by HPTLC shown in Fig. 2.3A. Fig. 2.3B shows concentration-dependant HPTLC pattern of standard withanolides as well as the withanolides from the root extract of the experimental samples. On the basis of $R_f$ values of 0.7, 0.58, 0.61, 0.68, and 0.79 for WS V, WF A, 1, 2 DWM, WN A and WN B, respectively, individual withanolides were identified. The concentrations of major withanolides are given in the Table 2.2.

It can be noted that deficiency of phosphorus, potassium and calcium had only marginal effect on the content of all withanolides. However, deficiency of nitrogen had significant deleterious effect. Thus content of WS V was totally abolished while there was an overall 35-36% reduction in the MW (Table 2.2).

2.3.6. Total withanolides (TW) Content by Spectrophotometric Analysis
TW content were reduced by 10.38%, 11.81%, 15.03% and 37.21% in Ca, K, P and N deficiency plants as compare to healthy plant (Table 2.2).

2.3.7. Molybdenum Content
It can be noted that nitrogen deficient plant had the lowest Mo content (7.02 ± 2.1 ppm) and potassium deficient plants had highest Mo content (33.5 ± 3.3 ppm) comparable to that of control (33.9 ± 1.6 ppm). Phosphorus deficient (13.1 ± 1.6 ppm) and Calcium deficient (17.1 ± 0.9 ppm) plants had intermediate values (Table 2.2).

Deficiency of Ca and P lowered the Mo content by 50 and 60% respectively while deficiency of N caused a significant 80% reduction. It is of interest to note here that despite 50 and 60% reduction in Mo content in Ca and P deficiency there was no significant decrease in the individual and TW contents (Table 2.2). However, when concentration of Mo was 7 ppm in nitrogen deficient
plants, the levels were affected significantly as pointed out above. Hence it maybe suggested that Mo levels of up to 13 ppm or so may be an absolute requirement for maintaining normal withanolide concentration; levels below this lead to adverse effect (Table 2.2).

With a view to elaborating the dependence of withanolide contents on Mo, the concentration of individual withanolides was plotted against Mo concentration from different 15 independent observations for control and nitrogen deficient groups. These plots are shown in Fig. 2.4. It is clear that for the control group a good correlation between withanolide content and Mo concentration was obtained; regression (R$^2$) values ranged from 0.76 to 0.99 (Fig. 2.4A). Even for nitrogen deficient group R$^2$ values ranged from 0.63 to 0.99 implying good correlationship (Fig. 2.4B).

### 2.3.8. Free Radical Scavenging Activity
Free radical scavenging activity of potassium deficient plant was highest (32.9 ± 1.7), whereas that for nitrogen deficient plants was lowest (24.7 ± 2.2) as compared to control plant (35.3 ± 1.1). Calcium deficient (31.7 ± 1.5) and Phosphorus deficient (30.5 ± 2.1) plants had intermediate values Table 2.2. The decrease in antioxidant activity seems to correlate with the reduction in TW and MW content in various deficiency conditions.
Fig. 2.3: (A) HPTLC chromatogram at 235 nm of major withanolides of Ashwagandha. (B) Image of HPTLC plate of Ashwagandha at 235 nm. 1-6 tracks for standards mixture; 9, 16 (Control); 8, 15 (-Ca); 7, 14 (-K); 11, 13 (-P), and 10, 12 (-N).
Table 2.2: Effect of macronutrient deficiency on withanoside V (WS V), withaferine A (WF A), 1, 2 deoxywithaastromonolide (1, 2 DWM), withanolide A (WN A), withanolide B (WN B), major withanolides (MW), total withanolides (TW), molybdenum (Mo) content and antioxidant activity of healthy (control), calcium deficient (-Ca), potassium deficient (-K), phosphorus deficient (-P) and nitrogen deficient (-N) Ashwagandha plant. Each value is mean ± standard deviation (SD) of fifteen independent samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>-Ca</th>
<th>-K</th>
<th>-P</th>
<th>-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS V (mg/g)</td>
<td>1.97 ± 0.6</td>
<td>1.54 ± 0.7</td>
<td>1.14 ± 0.8</td>
<td>1.54 ± 0.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>WF A (mg/g)</td>
<td>12.7 ± 2.7</td>
<td>10.25 ± 3.0</td>
<td>12.57 ± 3.4</td>
<td>9.25 ± 2.2</td>
<td>7.79 ± 2.2</td>
</tr>
<tr>
<td>1,2 DWA (mg/g)</td>
<td>1.52 ± 0.6</td>
<td>0.52 ± 0.3</td>
<td>0.76 ± 0.2</td>
<td>0.46 ± 0.8</td>
<td>1.31 ± 0.6</td>
</tr>
<tr>
<td>WN A (mg/g)</td>
<td>3.52 ± 1.2</td>
<td>4.72 ± 1.7</td>
<td>3.68 ± 1.7</td>
<td>4.31 ± 0.8</td>
<td>3.05 ± 1.2</td>
</tr>
<tr>
<td>WN B (mg/g)</td>
<td>2.97 ± 0.7</td>
<td>3.41 ± 0.7</td>
<td>2.57 ± 1.1</td>
<td>4.13 ± 1.4</td>
<td>2.59 ± 0.9</td>
</tr>
<tr>
<td>MW (mg/g)</td>
<td>22.9 ± 2.2</td>
<td>20.4 ± 3.8</td>
<td>20.7 ± 4.9</td>
<td>19.6 ± 3.1</td>
<td>14.7 ± 3.4</td>
</tr>
<tr>
<td>TW (mg/g)</td>
<td>55.9 ± 1.7</td>
<td>50.1 ± 1.1</td>
<td>49.3 ± 1.7</td>
<td>47.5 ± 4.7</td>
<td>35.1 ± 2.7</td>
</tr>
<tr>
<td>Mo (ppm)</td>
<td>33.9 ± 1.6</td>
<td>17.1 ± 0.9</td>
<td>33.5 ± 3.3</td>
<td>13.1 ± 1.6</td>
<td>7.02 ± 2.1</td>
</tr>
<tr>
<td>Reduction in MW (%)</td>
<td>--</td>
<td>11.08</td>
<td>9.68</td>
<td>14.17</td>
<td>35.75</td>
</tr>
<tr>
<td>Reduction in TW (%)</td>
<td>--</td>
<td>10.38</td>
<td>11.81</td>
<td>15.03</td>
<td>37.21</td>
</tr>
<tr>
<td>Free radical scavenging activity at 100μg (%)</td>
<td>35.3 ± 1.1</td>
<td>31.7 ± 1.5</td>
<td>32.9 ± 1.7</td>
<td>30.5 ± 2.1</td>
<td>24.7 ± 2.2</td>
</tr>
</tbody>
</table>
**Fig. 2.4:** Graph of regression ($R^2$) analysis of WS V, WF A, 1, 2 DWM, WN A and WN B with Mo content in the roots of control (A) and in -N plants (B). Each value is mean of fifteen independent samples.
Chapter 2: Influence of Nutrients Deficiency on Withanolides Content

2.4. Discussion

N, P, K and Ca are essential macronutrients for normal growth of the plants. It is reported that in healthy plants the average concentration of N, P, K and Ca were 15000 ppm, 10000 ppm, 10000 ppm and 5000 ppm respectively; while average concentration of Mo concentration was 50 ppm (Brady and Weil, 1999). The results of the present studies indicate that the macronutrient deficiency was observed in collected plant materials and deficiency symptoms can be characterized physical appearance in field. 45 kg N/ha and 26 kg P/ha have been reported to be optimum for normal growth of Ashwagandha (Nasira et al., 2012). Secondary metabolites differ with location, cultivation practices, soil nutrients, tissue type and varieties of Ashwagandha. Similar results were noted in the present studies. Root length and root diameter are important selection criteria for economic yield (Das et al., 2011). The medicinal properties of Ashwagandha are attributed to the withanolides content in the roots which are used in several medicinal formulations (Ali and Shuaib, 1997). Thus, critical concentration of macronutrients (N, P, K and Ca) and also possibly of Mo are important for the proper growth as well as withanolides content for commercial and therapeutic applications.

It is clear from the data presented (Table 2.2) that deficiency of Mo had dramatic effect on the withanolide contents; deficiency of other macronutrients had a marginal effect. The regression analysis studies showed that there was a positive correlation between withanolide contents and Mo content in the roots in control as well as N deficiency conditions (Fig. 2.4). Molybdenum functions as cofactor of many important plant enzymes involved in redox processes: nitrate reductase, xanthine dehydrogenase, aldehyde oxidase, and probably sulfite oxidase (Zimmer and Mendel 1999). There is no known transporter for Mo. It is believed that Mo is transported by sulphate (SO$_4^{2-}$) transporter. This assumption is
based on the fact that Mo has structural similarity to SO$_4^{2-}$. Transporter of SO$_4^{2-}$ is an energy-dependent process (Ferrari and Renosto, 1972). In other words it is a process of active transport. Therefore one would presume that Mo transport is also an energy-dependent active transport process. This would mean that the plants will accrue Mo by an energy-dependent process; concentration-dependent passive transport is not involved. In other words the plants will acquire the requisite quantity of Mo irrespective of its content in the soil. In this context it is of interest to note that the concentration of Mo in soil shows wide range of variation of 0.5 to 5 ppm; a tenfold variation (Sharma and Chatterjee, 1997).

It has been shown that uptake of Mo is interlinked with N metabolism; especially role of nitrate reductase has been emphasized (Axler et al., 1980; HamLin, 2006). Results of present studies are consistent with the above observation. The results would imply that only a severe deficiency of N would impair the withanolides content of the roots together with Mo content. The content of withanolides in roots is the most important criterion for therapeutic use. Hence it may be suggested that to the soils deficient in N manures rich in N e.g. urea, be added.

Thus it is important to maintain normal levels of these macronutrients N, P, K and Ca and especially of N for not only getting the good yield of the roots but also for getting good amounts of withanolide in the roots. It is also evident from the present work that N metabolism dependent Mo uptake influences the WF A accumulation, which is a major pharmaceutically important withanolide.
2.5. Conclusions
Regression analysis of the data emphasizes the importance of Mo in withanolide synthesis which is crucial for pharmacological properties. Micronutrient in the soil, where the herb is grown does influence the quality and quantity of the herbal product. Thus, these results bring out the importance of quality control of the herbal product at the level of plantation of the herb. For further studies plants with adequate quantities of macronutrients in the soil (control Group) were selected to prepare concentrated withanolide fraction which is described in the next chapter (Chapter 3).