Abstract

Withania somnifera is a predominant medicinal herb having economically valuable secondary metabolites and high reduction potential. Apart from being medicinal, *W. somnifera* also has hyper accumulation capability especially with heavy metals. Initially, metal bioaccumulation and metal reduction capability of field grown shoot tissues of *W. somnifera* was analysed using elemental analysis and extract based green synthesis of silver nanoparticles. The capability of *W. somnifera* to grow under the influence of heavy metal stress and accumulation of secondary metabolites is studied using *in vitro* shoot cultures. On confirmation of metal reduction capability, 45-days old *in vitro* shoot cultures were treated with different concentrations of silver nitrate and lead acetate salts at acute and chronic conditions. An Increase in biomass, primary and secondary metabolites (withanolides) was found to be accumulated in considerable amounts in metal salts treated *in vitro* shoots compared to *in vitro* control. Among the metal treated shoots, 1mM $AgNO_3$ treatment for 12 days period and 0.8mM PbAc treatment for 12 days period was selected as the optimum treatment conditions and selected for the further studies. Optimum $AgNO_3$ and PbAc treated shoots along with control shoots were analysed for its respective metal content using ICP MS analysis. The concentration of Ag in optimum $AgNO_3$ treated shoot is 50.8ppm and Pb in optimum PbAc treated shoot is 405ppm. The nature of Ag and Pb within the shoot was analysed by TEM with EDAX analysis. The presence of Ag and Pb nanoparticles in spherical and rod shape was confirmed. In addition, the neuroprotective activity of metal treated IVS along with field grown tissues of *W. somnifera* was studied using Parkinson's disease cell model (SH-SY5Y cells). Compared to field grown tissues, $AgNO_3$ treated IVS exhibited increased neuroprotective activity against rotenone toxicity. Molecular docking study was conducted to analyse the binding site of rotenone and selected withanolides in mitochondrial complex I protein. Multiple ligand simultaneous docking revealed a binding of rotenone with withaferin A leaves complex I protein uninhibited. Thus, from the current study, we conclude that $AgNO_3$ treated IVS along with increased withaferin A content has higher neuroprotective activity which may be used as a potential drug for toxins induced PD.

1.0 Introduction

Plants are the primary producers of our planet. They experience biotic and abiotic stresses on a daily basis in an open environment. Secondary metabolites have an important function in the defence mechanism of the plant. However, there are no reports on the role of secondary metabolites when plant is under normal condition per se. In the course of evolution, primary metabolic pathways of many plant species have been altered to synthesize secondary metabolites in order to withstand various external stressors. Several studies have reported that secondary metabolites protect plants from numerous external factors including pest resistance, biotic and abiotic stressors. Most of these secondary metabolites are restricted to particular family or species - specific. In addition, to elicit the production of secondary metabolites in plants, biotic and abiotic stressors have been used frequently. Metals are an important abiotic elicitor which acts as both stressors and nutrient (essential metals) to the plants. During metal elicitation, many plant species accumulate excess metal/ heavy metals as ions or nanoparticles in their vacuoles and other cell organelles.

Plants are able to take up heavy metals like Pb, Ag, As, Cr, Cd, although they have no biological functions within the plants. Heavy metal uptake via plant root is species and metal specific (Quah et al. 2015). Each metal has its unique ionic characteristic which alters the mode of adsorption by a plant, mode of transportation, translocation and even accumulation differs among plant species (Quah et al. 2015; Torrent et al. 2019).

Based on heavy metal absorption, plants are categorized as Accumulator, Excluder and Hyperaccumulator (Kushwaha et al. 2018; Egendorf et al. 2020). The accumulator plants have hyper-tolerance to the absorbed metal regardless of the tissue where heavy metals get accumulated (Pasricha et al. 2021). Further, in these plants, metals are absorbed by the root cells and gets translocated to shoots via heavy metal transporter system which may be species-specific. In the cases of Excluder, normally they absorb heavy metals from soil and accumulate or immobilize them within root cells or cell wall or other root cell organelles. Metal specific accumulator plants also known as hyperaccumulators which are capable of taking up heavy metals up to 1000-fold higher than those found in non-hyperaccumulator plants and also exhibits a high tolerance level (Rascio and Navari-Izzo 2011).

In the current study, we are going to investigate the uptake of heavy metals such as Lead and Silver by medicinal cum ayurvedic herb *Withania somnifera* commonly known as Indian Ginseng. *W. somnifera* is a medicinal plant native to India and few other Asian countries have plethora of pharmacological properties (Kaur et al. 2021). The pharmaceutical properties of *W. somnifera* are mostly determined by the presence of steroidal lactones (withanolides). In addition to the presence of withanolides, the major group of secondary metabolites, alkaloids, glycowithanolides, flavonoids and phenols are also present in *W. somnifera*. However, three important withanolides namely withaferin A, withanolide A and withanone are extensively studied and reported to have diverse therapeutic activity compared to others (Kaur et al. 2021).

W. somnifera has an accumulator capability that actively accumulates metal in its tissues with a high bioaccumulation coefficient even at a low soil concentration (Khan et al. 2007) which could be annulled via increased withanolides production; however, it is still unclear. Our previous studies mainly focused on the metabolite profiling among *in vitro* and field tissues of *W. somnifera* (Senthil et al. 2015; Purushotham et al. 2017). Field grown roots and leaves of *W. somnifera* are included in herbal formulations in traditional systems of medicine (Ven Murthy et al. 2010); nonetheless, it has a chance for more foreign/cross contaminants and high heavy metals risks. Therefore *in vitro* cultured tissues with similar metabolite content may increase its therapeutic value in the medicinal market. From our previous results, we observed that the secondary metabolite composition between *in vitro* shoot (IVS) is more similar to field-grown root (FGR) (Senthil et al. 2015; Purushotham et al. 2017). Although WFA was reported to be toxic at higher concentration, a minor quantity is still needed for *W. somnifera*'s therapeutic activity (Purushotham et al. 2017). Hence, *W. somnifera* FGR having minor WFA content is mostly used in the Ayurvedic formulations. Furthermore, the amount of WFA in FGR and IVS are nearly equal (Vinod et al. 2022; Natarajan et al. 2024a & 2024b). Based on this observation, we selected only *in vitro* shoot cultures for our studies.

Green synthesis of metal nanoparticles (MNPs) using plant (Luo et al. 2014; Kuppusamy et al. 2016; Kshtriya et al. 2021; Khan et al. 2023) or microbial extracts (Das et al. 2017) or organic agents (Sahu et al. 2016) is a rapidly advancing field in nanoscience. Green synthesized nanoparticles attract interest as they have plant metabolites as surface molecules which increases its stability and also acts as capping agent exhibiting less toxicity in environment (Sreekanth et al. 2016). In the field of green synthesis of nanoparticles, *in planta* formation of nanoparticles is also an emerging field of nanobiotechnology which emphasis live plant synthesis and accumulation of MNPs in the plant cell organelles (Parker et al. 2014; Schwab et al. 2015; Bhaskaran et al. 2019; Patil and Pawar 2019). Numerous studies have reported on metal stress tolerance (Cai et al. 2013) and resistance in plants (Rout et al. 2015; Mishra and Singh Sangwan 2018), accumulation of non-essential metal ions in plant tissues (Quah et al. 2015), *in planta* formation of MNPs in live plant tissues (Gardea et al. 2002 & 2003) and impact of engineered nanoparticles on plants or tissue cultures (Schwab et al. 2016; Quah et al. 2015). But the effect of *in planta* synthesized MNPs in their host plant including growth index, metabolomic changes, and determining the host plants' therapeutic potential has not been reported. In the present study, the *in planta* MNPs synthesizing capability of *in vitro* shoots (without root) and its potential therapeutic activity is reported for the first time.

Thus, the present study was formulated based on *W. somnifera*'s increased therapeutic and reduction potential (Natarajan et al. 2022). Two non-essential heavy metals such as silver and lead was selected. The metal salts were treated with *in vitro* shoot cultures and the concentration of metal salts were selected based on Indian metal contaminated and non-contaminated soil from the available reports (Parth et al., 2011; Kolesnikov et al. 2020; Patel et al., 2022).

Hypothesis

Null Hypothesis

The *in vitro* shoots of *W. somnifera* is susceptible to heavy metal stress and *in planta* metal reduction is not possible

Alternative Hypothesis

The *in vitro* shoots of *W. somnifera* exhibits tolerance to heavy metal stress and *in planta* formation of metal nanoparticles is possible which further increases its therapeutics.

To prove the alternative hypothesis the following objectives were framed

1. To confirm the metal accumulation and reduction potential of field tissues of *W. somnifera*
2. To study the effect of heavy metal salts on *W. somnifera in vitro* shoot growth and withanolides production.
3. To quantify and analyse the nature of bioaccumulated metal ions in *in vitro* grown shoots.
4. To evaluate the neuroprotective potential of *W. somnifera* tissues in Parkinson's disease cell model.