Manoj Kumar · Annamalai Muthusamy
Vivek Kumar · Neera Bhalla-Sarin
Editors

In vitro Plant Breeding towards Novel Agronomic Traits

Biotic and Abiotic Stress Tolerance

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Preface

Swelling demands for crop products prolong to rise strongly; agricultural productivity is threatened by various stress factors, often associated with global food for suitability and sustainability. To sustain and advance yield capacity, it is necessary to realize how plants respond to various stresses, and to use the hover knowledge in modern breeding programs. A good number of publications regarding molecular mechanisms associated with stress responses have been obtained from contemporary investigations using the model plant *Arabidopsis thaliana*. Molecular mechanism for stress amelioration is still in fancy where plant hormones, such as abscisic acid, jasmonic acid, and salicylic acid, have been shown to play key roles in defense responses against abiotic and biotic stresses. Epigenetic regulation at the DNA and histone level, and gene regulation by small non-coding RNAs also appear to be significant. Various approaches have been used for mutant screens, as well as next-generation sequencing approaches, to identify key factors and mechanisms of plant responses to the environment. However, it is often unclear to which extent the elucidated mechanisms also operate in crops.

This individual edition consequently seeks contributions to exposure of how crop plant species respond to various abiotic stresses, such as drought, heat, cold, flooding, and salinity, as well as biotic stimuli during microbial infections. The current edition welcomes reviews, perspectives, and original articles, and its focus is on our biochemical and molecular understanding of biotic and abiotic stress responses in crops, highlighting, among other aspects, the role of stress hormonal metabolism, fundamental mutagenesis, and changes in gene expression patterns and their regulation. Approaches and concepts to attain stress tolerance and to uphold yield permanence of agricultural crops during stress periods are of precise interest. These comprise perspectives on how knowledge from model plants can be utilized to facilitate crop-plant breeding and biotechnology.

The purpose of compendium is to conceptualize the innovative technology augmenting traditional-modern plant tissue culture, breeding, and markers approaches for novel agronomic traits with competency of biotic and abiotic stress tolerance. The book illustrates different evolutionary trends with modern concepts from different academic and research conditions to achieve sustainable tissue culture of ignored plants. The contributors highlight the contemporary themes, i.e., Breeding, Biotechnology and Molecular Tools and Volume, Agronomic, Abiotic and Biotic Stress Traits, and so on. The book contains 14 in-depth insight-based chapters from

learned researchers, academicians, and scholars who are working strategically for specific plant traits including improved nutritional and pharmaceutical properties as well as enhanced tolerance to insects, diseases, drought, salinity, and temperature extremes expected under predicted global climate change.

Ranchi, India — Manoj Kumar
Udupi, India — Annamalai Muthusamy
Dehradun, India — Vivek Kumar
New Delhi, India — Neera Bhalla-Sarin

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About the Editors

Manoj Kumar is an Associate Professor at the Department of Life Sciences, Central University of Jharkhand, Ranchi, India. Committed to research and development, Dr. Kumar holds a PhD in Plant Biotechnology and is involved in multidisciplinary research in the area of plant developmental biology, plant-microbe interaction and forest molecular genetics, including as a leader of a multidisciplinary research group. He is a referee for various journals, including *Phytoremediation* and the *Journal of Soil Sediments*.

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1

Withania somnifera (L.) Dunal: An Overview of Bioactive Molecules, Medicinal Properties and Enhancement of Bioactive Molecules Through Breeding Strategies

Poornima Poojari, Kodsara Ramachandra Kiran, Puthanvila Surendrababu Swathy, and Annamalai Muthusamy

Abstract

Withania somnifera is a very unique medicinal plant explored by number of orthodox medicinal systems such as Ayurveda, Siddha and Unani. It is commonly known as Ashwagandha, Indian ginseng and winter cherry, and finds its potential medicinal properties in the Ayurvedic Pharmacopoeia of India and Siddha Pharmacopoeia of India. The *Withania* genus is classified under the Solanaceae family and includes around 60 species, among which *W. somnifera* and *W. coagulans* are often mentioned in Ayurveda. Ashwagandha is a treasure house of widespread array of metabolites such as steroids, flavones, alkaloids, carbohydrates, glycosides, saponins, tannins, terpenoids and coumarin. Eight different polyphenols (five phenolic acids, vanillic, benzoic, p-coumaric, gallic and syringic acid, and three types of flavonoids, naringenin, catechin and kaempferol) were reported from Ashwagandha. Each part of this plant holds an assortment of different metabolites, and the metabolite concentrations vary among the different chemotypes. Ashwagandha has been used for a variety of ailment since a long time by traditional medicinal systems. It has been used for a range of diseases such as diabetes, emaciation, arthritis and rheumatoid arthritis-related inflammations, some kinds of seizures, diarrhoea, dermatitis and insect bite and specially used in the treatment of nervous disorder. The importance of Ashwagandha in medicinal treatments has attracted the attention of a large number of scientists; as a result, numerous experiments have been carried out, which have verified the therapeutic properties of Ashwagandha. Further, the

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efforts are being taken for the breeding strategies via conventional breeding as well as plant tissue culture techniques and gene transformation for the improvement of withanolide contents. This chapter gives an extensive insight on the various aspects such as basic introduction, classification, botanical description, bioactive molecules, medicinal properties and commercially available products. Furthermore, this chapter narrates the several breeding approaches for the improvement of withanolide contents of Ashwagandha.

Keywords
Ashwagandha · *Withania somnifera* · Medicinal properties · Bioactive compounds · Pharmacological effects · Improvement of withanolide content

1.1 Introduction

Withania somnifera (*W. somnifera*) is a very significant plant used in a number of orthodox medicinal systems like Ayurveda, Siddha and Unani. Ashwagandha, common name of *W. somnifera*, finds its mention both in the Ayurvedic and Siddha Pharmacopoeia of India (Umadevi et al. 2012). The use of Ashwagandha for its medicinal properties was preached by Punarvasu Atreya around 4000 years ago. Ayurveda described Ashwagandha as a rasayana herb, that is, an herb which has anti-ageing activity and can restore youthfulness by increasing physical strength and immunity. Rasayana also helps in improving memory and intelligence and relieves stress (Kulkarni and Dhir 2008). The renowned Indian literatures like *Charaka Samhita* and *Sushruta Samhita* have appreciated the importance of Ashwagandha for treating emaciation, rheumatoid arthritis-related inflammations and many other ailments. Ashwagandha is commonly used in India as a home therapy for alleviating hand and limb shivering of elderly people. It is also a very potent aphrodisiac. It is used to prepare a number of medicinal preparations, either alone or in combination with other herbs, for various types of nervous disorders as well. The leaves of Ashwagandha are useful in treating all types of skin lesions, boils, swelling, ulcers, pus formation and inflammation (Atal and Schwarting 1961).

1.1.1 Taxonomical Classification

The *Withania* genus is classified under the Solanaceae family and includes around 60 species of plants, among which *W. somnifera* and *W. coagulans* are often mentioned in Ayurveda. The taxonomical classification of Ashwagandha is as given below:

Kingdom: Plantae
Subkingdom: Tracheobionta
Super division: Spermatophyta
Division: Angiosperma

Class: Dicotyledons
Order: Tubiflorae
Family: Solanaceae
Subfamily: Solanoideae
Tribe: Physaleae
Subtribe: Withaninae
Genus: *Withania*
Species: *W. somnifera* Dunal

1.1.2 Botanical Description

The plant is a shrub growing to around 3–4 feet. It is usually erect and tomentose. The leaves of Ashwagandha are simple, ovate, exstipulate, glabrous and petiolate. The margin of the leaves is complete with acute to thick apex and cuneate or oblique base. The leaves are large and arranged alternate on the vegetative shoot but are opposite on floral branches. The flowers of Ashwagandha are pale green, inconspicuous, gamosepalous and arranged in cymose inflorescence. The flowers are characterized by the innate, oval anthers, epipetalous stamens arising from the petal base and slender filaments. Syncarpous gynoecium compelling of small swollen ovary with a long slender style. During the development of fruits, the calyx becomes enlarged, inflated and encloses the fruit. Fruits of this plant are berries having diameter of around 5 mm. The unripe fruits are green and turn red to orange-red on maturity. A single fruit encloses several small reniform seeds (Atal and Schwarting 1961; Mir et al. 2012).

1.1.3 Distribution and Cultivation

Ashwagandha wildly grows in dry regions and is wildly distributed in sub-tropical regions. It is found in Africa, Asia and Europe. It can be seen in African regions like Morocco, Congo, Egypt and South Africa, Middle Eastern region like Jordan as well as Asian regions like Afghanistan, India and Pakistan. In India, Ashwagandha is grown in Gujarat, Punjab, Uttar Pradesh, Maharashtra Haryana and Madhya Pradesh Rajasthan (Kokate 1996; Umadevi 2012,). Ashwagandha is an annual drought-tolerant plant. Semi-arid tropical areas with an average precipitation of 500–750 mm are appropriate for Ashwagandha as far as cultivation is concerned. Ashwagandha requires neutral to slightly basic soil with a pH of 7.5–8.0 and with a good drainage capacity as in sandy and sandy loam soil or red/black soil with light texture are advantageous for the growth of the plant. During the growing stage, dry season is necessary, and one or two late winter rains really favour the growth of the roots. The cultivation is easier and profitable since very less investment is required and the sale of roots fetch good price. The profit can be further boosted by selling leaves and the seeds. Hence it is cultivated on a large scale by small and marginal

farmers in drier areas especially in the areas in Karnataka, Rajasthan, Andhra Pradesh, Madhya Pradesh and other states in India (Rao et al. 2012).

1.2 Bioactive Compounds of Ashwagandha

Ashwagandha is a treasure house of wide-ranging metabolites such as steroids, flavones, alkaloids, carbohydrates, glycosides, saponins, tannins, terpenoids and coumarin (Singh et al. 2010a, b; Nasreen and Radha 2011) (Fig. 1.1). Alam et al. (2011) described the occurrence of eight different polyphenols (five phenolic acids: vanillic, benzoic, p-coumaric, gallic and syringic acid and three types of flavonoids: naringenin, catechin and kaempferol). Each part of this plant holds an assortment of different metabolites, and the metabolite concentrations vary among the different chemotypes of Ashwagandha (Kumar et al. 2007; Srivastava et al. 2018). Ashwagandha has physiological effects similar to *Panax ginseng*, with respect to the antistress activity, hence also named Indian ginseng (Grandhi et al. 1994). Currently, leaves are reported to harbour 62 and roots are known to harbour 48 major and minor primary as well as secondary metabolites, of which 29 are common major and minor primary as well as secondary metabolites in both leaves and roots. Among the secondary metabolites, withaferin A are common major bioactive molecules, whereas withanolide D are common minor bioactive molecules in both roots and leaves (Singh et al. 2015) (Fig. 1.2).

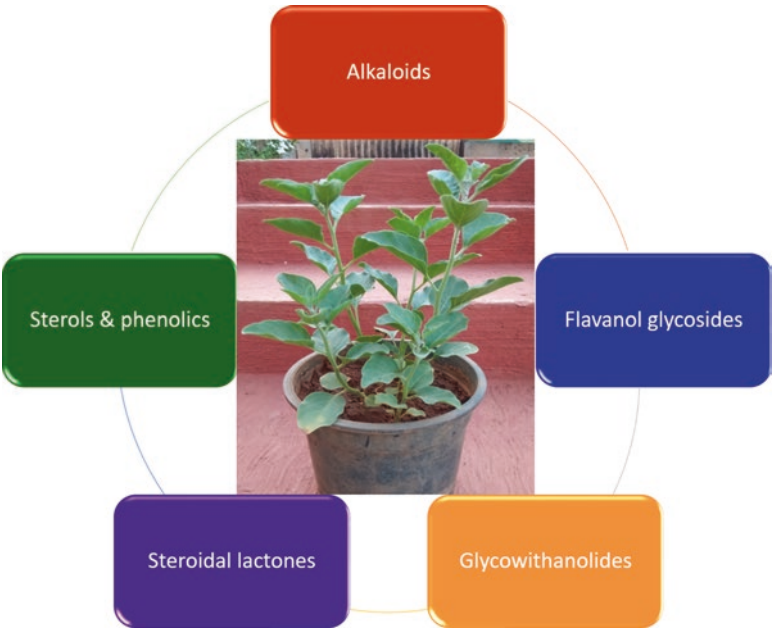

Fig. 1.1 Major groups of secondary metabolites from Ashwagandha

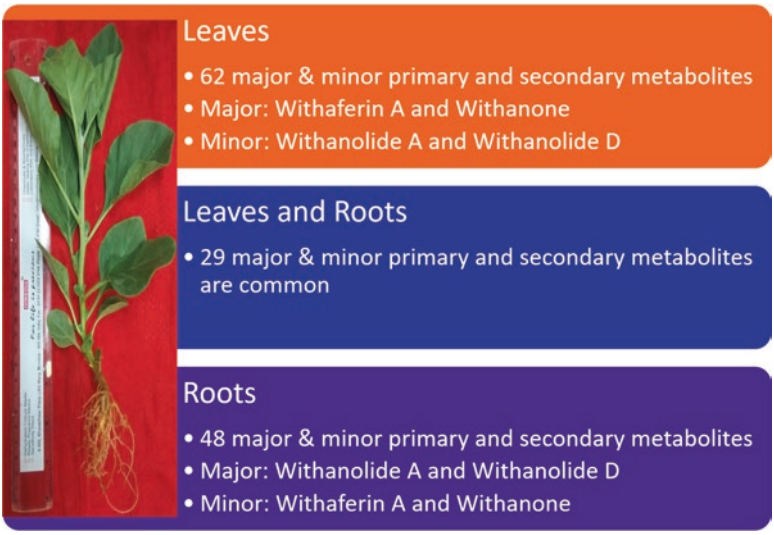

Fig. 1.2 Number of major and minor primary and secondary metabolites from Ashwagandha (Adopted from Singh et al. 2015 and modified)

1.2.1 Roots

Roots are therapeutically and traditionally most used part of the plant. Alam et al. (2011) determined the presence of catechin and benzoic acid in Ashwagandha by HPLC analysis of roots. Misra et al. (2008) were first to report the presence of stigmasterol glucoside, viscosa lactone B, α + β glucose, stigmasterol, β-sitosterol and β-sitosterol glucoside in the roots of Ashwagandha. Sharma et al. (2013a, b) analysed the alkaloid content of the roots of Ashwagandha with the help of GC-MS. They confirmed the presence of 17 alkaloids in the roots of Ashwagandha, which are withasomnine; somniferine; isopelletierine propanone; anaferine; anahygrine; pseudotropine; Iron-pseudotropine; withanine; 1-[(5-Nitro-2-furfurylidene)amino]; oxacyclohexadecane-2,13-dione,13-oxime; scopoletin; Ashwagandhine; 2,4-imidazolidiendione1-{(5-nitro-2-furanyl)methylene]amino}. The alkaloid withasomine and anaferine are reportedly the chief alkaloids the roots of Ashwagandha (Sharma et al. 2013a, b). Misra et al. (2012) isolated stearic fatty acids, sitostanone, oleanolic acid, octacosane, stigmasterone, oleic acids, 1,4-dioxane derivative, stigmasterol and ergosterol from Ashwagandha root extracted using n-hexane. According to Chaurasiya et al. (2008), major withanolides of the roots are withaferin A, 17-hydroxy-27-deoxy withaferin A and 17-hydroxy withaferin A. They reported the presence of 27-deoxy withaferin A and 27 hydroxy withanolide B in the roots as well albeit in lower amount. Two new compounds 6α-hydroxy-5,7α-epoxy as functional groups and 16β-acetoxy-17 (20)-ene from the Ashwagandha roots were isolated by Misra et al. (2008).

Roots of Ashwagandha also contain acylsterylglucosides like sitoindoside VII and sitoindoside VIII; (20S, 22R)-5a,27-dihydroxy-6a,7a-epoxy-1-oxowitha-2,24-dienolide; (20S,22R)-3 alpha, 6 alpha-epoxy-4 beta, 5 beta, 27-trihydroxy-1-oxowitha-24-enolide; (20S,22R)-4b,5b,6a,27-tetrahydroxy-1-oxowitha-2,24-dienolide; physagulin D; withacoagin; withanolide D; lycium substance B; and withanoside II–XI (Bhattacharya et al. 1987, Zhao et al. 2002).

1.2.2 Leaves

Singh et al. (2010a, b) identified amino acids like alanine, aspartate, asparagines and choline from leaves. Nema et al. (2013) profiled various phytoconstituents such as starch, amino acids, carbohydrate, protein, flavonoids, tannins, alkaloids, oxalic acid, steroids, phenolic compounds and inorganic acids from hydroalcoholic extract of Ashwagandha leaves. Bashir et al. (2013) detected the flavonoids, steroids, alkaloids, saponins and tannins in the methanolic crude extract of Ashwagandha leaves. They also isolated three flavonoids, namely, 5, 7, 4′-triahydroxy-methyl-3-O-galactosyl flavonol, 7, 3′, 4′-trihydroxy flavone-3-O-rhmnosyl and quercetin -3-O-galactosyl from the leaf extract and characterized their structures based on NMR, IR and MS spectroscopy.

Jayaprakasam and Nair (2003) discovered four novel withanolide glycosides, namely, 27-O-β-D-glucopyranosyl viscosalactone B; physagulin D (1 → 6)-β-d-glucopyranosyl-(1 → 4)-β-d-glucopyranoside; 4,16-dihydroxy-5β,6β-epoxyphysagulin D and 27-O-β-D-glucopyranosyl physagulin D; and a novel withanolide, viz. 4-(1-hydroxy-2,2-dimethylcyclo-propanone)-2,3-dihydrowithaferin A from Ashwagandha leaves. They also reported seven known withanolides withaferin A, viscosalactone B, sitoindoside IX, physagulin D and withanoside IV, 2,3-dihydrowithaferin A and 27-desoxy-24,25-dihydrowithaferin A from Ashwagandha leaves.

The leaves of Ashwagandha also contain chlorinated withanolides, namely, 6α-chloro-5β,17α-dihydroxywithaferin A and withanolide Z. Furthermore, leaves also contain withaferin A, withanone, 6α-chloro-5β-hydroxywithaferin A, withanolide B, (22R)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith-24-enolide, withanolide A, withanoside IV, 2,3-dihydrowithaferin A, 3-methoxy-2,3-dihydrowithaferin A, 2,3-diehydrosomnifericin, withanoside X and 27-hydroxywithanolide B (Pramanick et al. 2008; Tong et al. 2011).

1.2.3 Fruits

Bhatia et al. (2013) determined different aromatic and aliphatic amino acids, fatty acids, phenolic acids, organic acids, sterols, tocopherols, polyols, sugars and withanamides from the fruits of Ashwagandha using ^{1}H NMR spectroscopy and GC-MS. These compounds are mainly involved in the different metabolic pathways like shikimic acid, mevalonate (MVA), non-mevalonate (DOXP) and

phenylpropanoid pathway. The metabolite concentrations varied among the fruits of different chemotypes of Ashwagandha. Lal et al. (2006) reported two withanolides, viz. iso-withanone which has an unusual 17α-oriented side chain and 6α,7α-epoxy-1α,3β,5α-trihydroxy-witha-24-enolide possessing an 1α,3β -dihydroxy group for the first time from the fresh berries of a plant in India. These berries also contain coumarins like scopoletin and aesculetin and triterpene like β-amyrin (Abou-Douh 2002). Reportedly the fruits also contain tocopherols like α-tocopherol and β-tocopherol and phytosterols like cholesterol, β-sitosterol, campesterol and stigmasterol (Bhatia et al. 2013; Abou-Douh 2002). Fruits of Ashwagandha contain withanolide A and withanone in substantial amount. Other withanolides present in the fruit are 27-deoxy withaferin A, 27 hydroxy withanolide B and 27-hydroxy withanolide A (Chaurasiya et al. 2008; Lal et al. 2006). Abou-Douh (2002) isolated two novel withanolides 5β, 6α, 14α, 17β, 20β-pentahydroxy-1-oxo-20 S, 22R-witha-2, 24-dienolide and 6α, 7α-epoxy-5α, 14α, 17α, 23β-tetrahydroxy-1-oxo-22R-witha-2, 24-dienolide from the Ashwagandha fruit grown in Southern Egypt. They also contain nine different withanamides, named withanamide A to withanamide I; this class of compound has a unique chemical structure, consisting of long-chain hydroxyl fatty acid moieties, glucose and serotonin (Jayaprakasam et al. 2004).

1.2.4 Bark

Five new withanolides, viz. somnifera withanolide, withanolide, somniwithanolide, withasomniferanolide and somniferanolide, were isolated from the bark of Ashwagandha grown at South Delhi region and characterized through spectroscopic and phytochemical techniques (Ali et al. 1997).

1.2.5 Commercial Products

Currently more than 24 products are available in the market as Ashwagandha as a sole or one of the ingredients. Ashwagandha products are used in wide range from overall health to specific health for organs and glands (Table 1.1).

1.3 Pharmacological Effects of Ashwagandha

Ashwagandha has been used for a variety of ailment since a long time by traditional medicinal systems. It has been used for a range of diseases such as diabetes, emaciation, arthritis and rheumatoid arthritis-related inflammations, seizures, diarrhoea, dermatitis and insect bite and specially used in the treatment of nervous disorder (Verma and Kumar 2011). The importance of Ashwagandha in medicinal treatments has attracted the attention of a large number of scientists; as a result,

Table 1.1 Commercially available products of *Withania somnifera*

S. No.	Product name	Manufacturer	Benefits	References
01	Adrenal Health	Gaia Herbs	This mix of herbs nourishes the adrenals, enabling the body to adapt to stress	https://www.gaiaherbs.com/products/detail/777/Adrenal-Health-Daily-Support
02	Ashwagandha Root	Gaia Herbs	This includes single herb, Ashwagandha, and helps nourish and restore the optimal nervous and immune system, energy levels and overall immune function	https://www.gaiaherbs.com/products/detail/662/Ashwagandha-Root
03	SleepThru	Gaia Herbs	This blend of herbs supports restful sleep and healthy adrenal function	https://www.gaiaherbs.com/products/detail/745/SleepThru
04	Stress Response	Gaia Herbs	This formula of herbs supports a healthy response to stress	https://www.gaiaherbs.com/products/detail/77/Stress-Response
05	Thyroid Support	Gaia Herbs	A combination of Ashwagandha and other herbs, this formulation supports normal thyroid hormone production, which in turn helps maintain proper weight, neuromuscular and cardiovascular health	https://www.gaiaherbs.com/products/detail/81/Thyroid-Support
06	Ashwagandha	Himalaya Herbals	Relieves stress by acting on adrenal glands by normalizing cortisol levels and eases sleeplessness	http://himalayausa.com/products/best-sellers/organic-ashwagandha/
07	Anti-Stress Massage Oil	Himalaya herbals	Useful in overcome stress and fatigue	http://www.himalayaherbals.com/products/healthcare/anti-stress-massage-oil.htm
08	Ashwagandharishta	Baidyanath Ayurved	This liquid medicine helps in alleviating all kinds of vata-related diseases. It is beneficial in controlling diabetes, digestive problems, fatigue and fever. It helps strengthen immunity and memory. It is useful in treatment of men problems like impotency and erectile dysfunction	https://www.baidyanath.com/product/ashwagandharishta/

09	Ashwagandha Capsules	Planet Ayurveda	This medicine, prepared from Ashwagandha root extract, is useful in treating debilitating conditions, fatigue, stress, anxiety, palpitation, ageing, nervous breakdown, neuropathy due to diabetes, chronic fatigue syndrome and loss of weight due to cancer, diabetes or any other reason	https://www.planetayurveda.com/ashwagandha_uses.htm
			Strengthens and relieves muscles. It is a good nerve tonic. It can be given to growing children, as well as to old age people	
10	Male Support	Planet Ayurveda	Improves general health. Beneficial for erectile dysfunction, premature ejaculation, low sperm count, general physical and sexual weakness, and infertility in men	https://www.planetayurveda.com/male-support-formula.htm
11	Aller-G Care Capsule	Planet Ayurveda	Best anti-allergic and anti-inflammatory herbs	https://www.planetayurveda.com/allergformula.htm
12	Aamvatantak Churna	Planet Ayurveda	This is a blend of unique herbs, which also has Ashwagandha as one of its ingredients. Used for controlling rheumatoid arthritis and its symptoms like stiff joints, inflammation, swelling and joint pain	https://www.planetayurveda.com/aamvatantak.htm
13	Total Heart Support Capsules	Planet Ayurveda	A combination of herbs with Ashwagandha as one of its ingredients; this medication is effective in controlling high cholesterol, blocked coronary arteries, congestive heart failures. This strengthens failing heart muscles, eases breathlessness and cleanses blocked arteries. Useful in post-myocardial infarction support and congestive heart failure	https://www.planetayurveda.com/total-heart-support.htm
14	Stress Support Capsules	Planet Ayurveda	Corrects vata imbalances due to stress	https://www.planetayurveda.com/stress-support.htm
15	Dabur Ashwagandha Churna	Dabur India Limited	Improves stamina and energy. Relief from stress, weakness	
16	Dabur Ashwagandharishta	Dabur India Limited	This contains other medicinal plants like Ashwagandha, Mushali, Manjishtha, Haritaki and Nisha. These ingredients together help treating depression and anxiety, improving memory, calming nerves and strengthening digestive system	https://www.dabur.com/in/en-us/ayurvedic-herbal-products/dabur-ashwagandharishta

(continued)

Table 1.1 (continued)

S. No.	Product name	Manufacturer	Benefits	References
17	Sona Chandi Chyawanprash Plus	Zandu	This contains other ingredients like Bala, Guduchi, Haritaki, Jyotishmati, Pippali, Punarnava, Yastimadhu and Amla Pishti, along with Ashwagandha, which are known for their immunity-boosting properties. This preparation improves memory and learning ability and body immunity and controls frequency of cough, cold and allergies	http://www.zanduayurveda.com/products/71/zandu-sona-chandi-chyawanplus.php
18	Vigorex	Zandu	Ashwagandha is one of the ingredients of this product. This product is used to enhance stamina and energy	http://www.zanduayurveda.com/products/70/vigorex.php
19	Zandu Pancharishta	Zandu	Ashwagandha is one of the ingredients of this tonic. This helps in building the entire digestive system and building digestive immunity and reduces problems like acidity, gas, indigestion, flatulence and constipation	http://www.zanduayurveda.com/products/29/zandu-pancharishta.php
20	Zandu Kesari Jivan	Zandu	Also contains Kesar, fresh Amla, exotic herbs, spices and trace minerals Makes one physically strong and keeps youthful vigour intact	http://www.zanduayurveda.com/products/27/zandu-kesari-jivan.php
21	Brento	Zandu	It is a brain tonic which restores brain energy. Useful in treating mental debility, cognitive dysfunction and other mental complications	http://www.zanduayurveda.com/products/31/brento.php
22	Ashwagandha Capsules	Dehlvi Naturals	Beneficial for people with mental problems, arthritis, asthma, sexual problems, bradycardia, bronchitis, cancer, convalescence, dyspepsia, general debility, hydrospermia, hypertension, infertility, insomnia, lack of appetite, leucorrhoea, lumbago, polyuria, premature ageing, stress, suppressed post-partum lactation	http://www.dehlvi.com/medicine-Ashwagandha_Capsules-view-598.html
23	Ashwagandha Ras	Dehlvi Naturals	Helps in the treatment of anxiety, arthritis, convalescence, depression, erectile dysfunction, exhaustion, fatigue, general debility, infertility, lack of vigour, lumbago, rheumatism, rheumatoid arthritis, seminal debility, senile debility, sexual debility and stress	http://www.dehlvi.com/medicine-Ashwagandha_Ras-view-636.html
24	Ashwagandha Tea	Buddha Tea	Ashwagandha tea helps in de-stressing and improving memory	https://www.buddhateas.com/ashwagandha-tea.html

numerous experiments have been carried out to verify the therapeutic properties of Ashwagandha.

The in vitro studies have demonstrated that Ashwagandha possesses anxiolytic (Andrade et al. 2000), cytoprotective (Thiagarajan et al. 2003), antifungal (Singh et al. 2010a, b), antioxidant, pesticidal (Gupta and Srivastava 2008) and antibacterial properties (Mehrotra et al. 2011). Mahdi et al. (2009) stated that this plant could improve fertility by improving sperm quality and stress-related infertility. Various studies on animal models demonstrated that the extracts of Ashwagandha improve sexual health (Borde et al. 2008) and exhibit cardioprotective (Gupta et al. 2004), immunomodulatory (Gupta et al. 2006), hypocholesteremic (Visavadiya and Narasimhacharya 2007), antidiabetic and anti-ageing (Babu et al. 2007), hypoglycaemic (Udayakumar et al. 2009) and antilipidemic effects (Khursheed et al. 2010). The main pharmacological activities of the Ashwagandha are described separately.

1.3.1 Antistress and Antioxidant Property

Various studies have been showed to authenticate the antioxidative property of Ashwagandha, and it has been observed to influence both the non-enzymatic and enzymatic antioxidants. Ashwagandha restores the activity of antioxidant enzymes, viz. glutathione reductase (GR), glutathione- S-transferase (GST) superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) to the near normal levels and decreases lipid peroxidation (LPO) (Anwer et al. 2012; Kumar and Kumar, 2008). Bhattacharya et al. (2001) demonstrated that the glycowithanolides present in the Ashwagandha could normalize the activity of enzymatic antioxidants like CAT, SOD, GPX and LPO in the frontal cortex and striatum of the rat brain with induced chronic footshock stress. Anwer et al. (2012) noted the augmentation in the activity of antioxidant enzymes in the type 2 diabetic rats when administered extract of Ashwagandha, which results in the reinstatement of the form and structure of pancreatic β-cells close to normal form. The intake of Ashwagandha extracts also helped in bringing the blood glucose, tissue LPO levels and glutathione (GSH) contents to a near normal state.

1.3.2 Aphrodisiac Properties

Mahdi et al. (2009) suggested that Ashwagandha could help the male with infertility due to stress. The normozoospermic males suffering from infertility, due to heavy smoking, psychological stress or/and with other unknown reason, witnessed a general improvement in semen quality with a decrease in the stress. The partners of 14% of the individuals could even conceived after the treatment and decrease in the lipid peroxidation and increase in enzymes of antioxidant system, antioxidant vitamins and fructose in the seminal plasma was noted. Ashwagandha controlled

protein carbonyl content and enhanced the sperm quality. The treatment stabilized the levels of sex hormones like testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin in infertile subjects (Ahmad et al. 2010). In a study, Shukla et al. (2011) observed the reduced apoptosis and intracellular level of ROS in spermatozoa and stabilized level of the important metal ions such as Cu^{2+}, Zn^{2+}, Fe^{2+} and Au^{2+} in seminal plasma of infertile individuals after treatment with Ashwagandha. Administration with Ashwagandha improved the levels of citrate, lactate, GPC and aliphatic amino acids like alanine and aromatic amino acids like histidine and phenylalanine in seminal fluid, and the semen quality was found to be recovered in Ashwagandha-treated men (Gupta et al. 2013). The intake of the plant was noticeably increased sex hormones and gonadotropins especially testosterone, oestrogen and luteinizing hormone even in the male rats addicted to morphine. Morphine consumption results in a number of sexual and fertility problems. Intake of Ashwagandha also increased the level of progesterone which stimulates most sex hormones in addicted male rats (Rahmati et al. 2016).

1.3.3 Antidepression Properties

The roots of Ashwagandha have been termed as rasayana herb in Ayurveda, which has a positive effect on the physical as well as mental health. Bhattacharya et al. (2000b) observed that the anxiolytic effect of the glycowithanolides was comparable to that of lorazepam as studied by the tests like elevated plus maze, feeding potential in unacquainted environment and social interaction. Glycowithanolides could also reduce the anxiety by reducing the concentration of tribulin which is a well-known marker of stress and anxiety, like lorazepam. The bioactive component also displayed antidepressant effect in the forced swim-induced 'behavioural despair' and 'learned helplessness' tests. Hence Bhattacharya et al. (2000b) put forward the idea that the glycowithanolides from Ashwagandha root could help in the state of anxiety and depression. Nonetheless, Ashwagandha root extract improved the open field behaviour and emotional stability in normal rats as well. It also enhanced the functional sensitivity of 5 HT2 receptors in the brain and a reciprocal subsensitivity of the 5HT1A receptors. Chronic Ashwagandha treatment also resulted into adaptive supersensitivity of the postsynaptic 5HT2 receptors in the brain. The observations were similar to the ones caused by the chronic electroconvulsive therapy treatment and several antidepressant drugs (Tripathi et al. 1998). Jayanthi et al. (2012) noted the dose-dependent reduction in immobility time in forced swim test (FST) and tail suspension test (TST) in rat administered with fat extract of Ashwagandha, Ayurvedic formulation Ashwagandha ghrita (AGG). In the Reserpine test, the scores of the ptosis, catatonia and sedation were significantly improvised in the groups treated with AGG and combination of AGG with Imipramine. These observations supported the use of Ashwagandha as potential adjuvant in depression disorders.

1.3.4 Neurological Effects

Ashwagandha extracts could suppress the release of corticosterone in the mice, the chemical which can induce neurotoxicity (Bhatnagar et al. 2009; Huang et al. 2009). This in turn activates choline acetyltransferase causing an increase in the level of serotonin in the hippocampus and inhibition of NADPH-d activity of neuronal nitric oxide synthase (nNOS). The key fundamental mechanism of the neuroprotective effects of Ashwagandha can be accredited to its involvement in the selective inhibition of nNOS and variations of definite neurotransmitter systems. Attari et al. (2016) presented the fact that probably the NO (nitric oxide) does not mediate this beneficial effect and Ashwagandha may affect other neurochemical systems and pathways. They found that Ashwagandha extract dose dependently decreased the immobility time comparable to fluoxetine (20 mg/kg). Ashwagandha also lowered the immobility measure in TST. These effects were not related to change in locomotor activity. L-NAME (N omega-nitro-L-arginine), the NOS inhibitor, did not influence the effect of the extract on the behavioural tests. Ashwagandha root exhibited a cytoprotective effect on separated PC12 cells against cytotoxic effects of both H_2O_2- and Ab(1–42). Ashwagandha extract could prevent the maturation of amyloid-β fibrils in vitro, which is the first significant step towards formation of amyloid plaque in vivo (Kumar et al. 2012). Kurapati et al. (2013) further perceived that the Ashwagandha extract could neutralize the toxicity mediated by β-amyloid and HIV-1 Ba-L(clade B) infection in the human neuron-derived cell line -SK-N-MC. Furthermore, Sehgal et al. (2012) put forth that Ashwagandha extract could fix the behavioural abnormality, accumulation of oligomers of beta-amyloid peptides (Aβ) and plaque formation in the brains of APP/PS1 Alzheimer's disease transgenic mice. They also put forth exceptional observation that the treatment with Ashwagandha caused decrease in brain Aβ monomer accompanied with an increase in plasma Aβ. They also noticed that the level of low-density lipoprotein receptor-related protein (LRP) and Aβ-degrading protease neprilysin in brain microvessels and plasma sLRP increased before the transfer of the Aβ peptides into plasma. Ashwagandha could be used to enhance the immediate and general memory in people with mild cognitive impairment was confirmed in a pilot study in India. It was also noticed that it could improve executive function, attention and information processing speed (Choudhary et al. 2017). The neuroprotective effect of the plant extract was studied in human cultured neuron model, which was injured to represent mildly traumatized brain injury. It was observed that there was an increase in the neurites length and neuronal survival (Saykally et al. 2017). Among the different constituents of the Ashwagandha components, the study by Pandey et al. (2018) specifically worked on withanone and found that in Wistar rats, it could boost the cognitive skills by inhibiting amyloid β-42 and controlled the levels of pro-inflammatory cytokines and eased the pathophysiology of the disease. In a randomized clinical trial, it was confirmed that Ashwagandha extract when used as adjunctive treatment benefits the patients with recent exacerbation of schizophrenia, along with decrease in the level of stress in the patients (Chengappa 2018).

1.3.5 Cytoprotective Activity

Bhattacharya et al. (2000a) observed that the treatment of equimolar concentrations of sitoindosides VII–X and withaferin A have stabilized the hepatic lipid peroxidation and levels of specific aminotransferase and lactate dehydrogenase in serum of rats with induced hepatotoxicity. Hence signifying the potential use of Ashwagandha in the treatment of heavy metal as well as other toxins induced hepatic dysfunction as the Ashwagandha extract is rich in antioxidant glycowithanolides. Sumantran et al. (2007) verified the inhibition of gelatinase activity of type 2 collagenase and theory by exhibiting chondroprotective action on diseased osteoarthritic cartilage by aqueous extract of Ashwagandha. Nakajima et al. (2011) observed that Ashwagandha extract alleviates endothelin-1(EDN1)-mediated pigmentation by restraining EDN1-activated protein kinase C (PKC) activity. Withaferin A causes the downregulation of the phosphorylation of the kinases like Raf-1, MEK, ERK, MITF and CREB 15 min after EDN1 treatment. Since the phosphorylation of Raf-1 by PKC activity can be downregulated by withaferin A, these findings indicate that hyperpigmentary disorders can be treated with the help of an antioxidant-rich Ashwagandha extract.

1.3.6 Antitumour Properties

Many researchers have put light on the antitumour and proliferation inhibitory effect of Ashwagandha on the cancer cells both in cell lines as well as animal model system. Osman et al. (2012) reported the cytotoxic and proliferation inhibitory effect of Ashwagandha extract on MCF 7, a human breast cancer cell line, with IC50 0.86% as measured by Trypan blue and MTT assay. Oza et al. (2010) investigated the antitumour effect of purified L-asparaginase from Ashwagandha fruits which showed antitumour activity at very low doses, and subsequently Ashwagandha is explored as a potent source of L-asparaginase. L-Asparaginase from Ashwagandha is known to have structural and functional similarity with bacterial L-asparaginases EC-2. Kataria et al. (2011) showed that the Ashwagandha could be used as safer complimentary therapy for glioma as the aqueous extract of Ashwagandha leaves exhibited proliferation inhibiting, differentiation inducing and anti-metastasis effect. Khazal and Hill (2015) reported the antitumour effect of Ashwagandha root extract on the xenografts model of breast cancer cell line MDA-MB-231 in nude mouse. The substantial increase in the sub-G1 phase cells and cytotoxic effect on MDA-MB-231 cells in dose-dependent manner indicate the potential complimentary therapy for breast cancer using Ashwagandha extract. In totality Ashwagandha root extract could inhibit the proliferation of breast cancer cells in vitro as well as in vivo, accompanied by significant downregulation of CCL2, a chemokine. The study by Henley et al. (2017) revealed that Ashwagandha root extract could ‘prime’ the cancer cells, specifically HT-29 colon cells, to the chemotherapy. This increased the effectiveness of the chemotheraupetic agent used (cisplatin) through enhancement of the mitochondrial dysfunction.

1.3.7 Antimicrobial Activity

The extracts of Ashwagandha have been demonstrated to have antifungal, antibacterial and pesticidal properties. Khan and Nasreen (2010) found that the protein fraction of Ashwagandha exhibited higher inhibition of mycelia growth of *Bipolaris oryzae* and *Colletotrichum lindemuthianum* than nonprotein fractions. Similarly, Singh et al. (2010a, b) used the solvent extract of Ashwagandha for agar diffusion test against a number of bacteria. The Ashwagandha extract using ethyl acetate and hexane exhibited higher inhibition zone against *Aspergillus niger*, whereas methanolic extract inhibited the growth of *Fusarium oxysporum* and *A. flavus*, while the growth of *F. moniliformis* was inhibited by aqueous extract. Singariya et al. (2012) also reported that Ashwagandha extracts had antimicrobial activity against important human pathogens. Furthermore, the polar and non-polar solvent extracts of immature and mature fruit and calyx of Ashwagandha were screened for their antimicrobial activity against *Bacillus subtilis* (Gram-positive bacteria), *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (Gram-negative bacteria) and *A. flavus* (fungus) by agar diffusion test. Chloroform extract of Ashwagandha calyx revealed maximum activity against *B. subtilis*. Similarly Gupta and Srivastava (2008) found that different extracts of (aqueous suspension and ether) various parts of Ashwagandha showed varying effect on the adult mortality of pest *Callosobruchus chinensis* L. The ether extract resulted in higher adult mortality followed by aqueous extract. Recently there have been increased cases of resistance of pathogenic bacteria to antibiotics, pushing the researchers to find herbal alternatives. Bisht and Rawat (2014) noticed that the methanolic leaf extract of Ashwagandha could actively hinder the pathogens, including a total of 20 isolates including methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *S. aureus*, *Enterococcus* and *Streptococcus* spp. Datta et al. (2011) also reported growth inhibitory effect of Ashwagandha root extracts against all the multidrug-resistant strains of *S. aureus* isolated from local and patient sources. Bokaeian et al. (2015) remarked that Ashwagandha extracts could inhibit the drug-resistant *Escherichia coli* strains collected from patients with infections in urinary tract.

1.4 Breeding Strategies for Improvement of Withanolide Content in *W. somnifera*

1.4.1 Plant Cell, Tissue and Organ Culture

Many researchers have established protocols for propagation of Ashwagandha and analysed the amount of withanolides in the cultured plants and different parts of the plant. Roja et al. (1991) produced Ashwagandha plantlets and callus from axillary meristems. They observed that the amount of withanolides varies at the different morphogenetic stages. At callus stage they did not detect withanolides, whereas the multiple shoot cultures could synthesize a substantial amount of withanolides. They also noticed that the amount of withanolides varied with different hormonal

combinations, illustrating the importance of hormones in the biosynthesis of withanolide. Further Sangwan et al. (2007) described that the genotype of the plant along with the hormonal combination, influenced the withanolide A accumulation in shoot cultures. They established the shoot cultures from nodal segments of Ashwagandha plants from two experimental lines, i.e. *RS-Selection-1* and *RS-Selection-2*, using different combinations of plant growth regulators. Using [2-14C] acetate as a precursor, they also demonstrated that the in vitro shoots synthesized withanolide A de novo. Awasthi et al. (2008) reported elevated production of withanolide A, withanolides and withaferin A, in the plantlets regenerated in vitro from axillary bud explants by using additional carbohydrates in the rooting media. Multiple shoots were developed from axillary bud explants on Murashige and Skoog (MS) media fortified with 0.5 mg/1 of 6-benzylaminopurine (BAP) and 1.0 mg/1 of KN. The shoots proliferated on 3/4 MS medium complemented with BAP and KN (0.1 mg/l and 0.05 mg/l, respectively) with mean of 8 shoots per explants. The shoots were excised and subcultured onto ½ MS with 2.0 mg/L IBA with additional carbohydrate (4% sucrose +2% mannitol) for rooting. The rooted shoots thus obtained were then transferred to the soil. It was reported that the roots of these micropropagated plant contained more withaferin A (0.047% w/w) and withanolide A (0.0401% w/w), respectively, as compared to the seed raised plants (0.0379% w/w and 0.0386% w/w, respectively). A group of scientists demonstrated that the concentration of macroelements present in the media, too, influenced the accumulation of withanolides A and biomass in the cell suspension and adventitious root suspension culture of Ashwagandha. They varied the concentration of calcium chloride, potassium sulphate, ammonium nitrate, magnesium sulphate and potassium nitrate and strength of nitrogen source [NH_4^+/NO_3^-]. In the cell suspension culture, maximum of withanolide A production (4.36 mg g^{-1} DW) was achieved with 2.09 KNO_3 in media, and both the parameters were increased when nitrate concentration was higher than ammonia. In case of adventitious root suspension culture, maximum biomass (127.52 g/L FW and 12.45 g/L) was recorded in 0.5x concentration of NH_4NO_3, and maximum accumulation (14.00 mg/g DW) of withanolide A was observed with 2.0x KNO_3. Higher biomass of Ashwaganda was noted when NO_3^- was greater than the NH_4^+, whereas absence of NH_4^+ was favor withanolide A production. Similarly, the treatment ratio of NH_4^+/NO_3^- at 0.00:18.80 mM showed maximum production of withanolide A (Murthy and Praveen 2011; Murthy and Praveen 2012).

1.4.2 Biotic and Abiotic Elicitors

Despite the optimization of various media constituents, the amount of withanolides produced was not as high. Many researchers successfully used the elicitors to further increase the content of withanolides in Ashwagandha through in vitro culture. Elicitors are microbial, physical or chemical factors which invoke physiological and morphological response in plants. Elicitation is a technique to fortify survival endurance and competitiveness of plants by enhancing the synthesis of secondary

metabolites. The secondary metabolites production was successfully achieved in many medicinal plants by using the elicitors (Patel and Krishnamurthy 2013).

The elicitation of withanolides production in Ashwagandha was first demonstrated by Ciddi (2006). They used biotic elicitor salacin, methyl jasmonate (MJ) and arachidonic acid for the elicitation experiments in the cell suspension culture. Salacin (750 mM) enhanced the synthesis of withaferin A by 50-fold (25 ± 2.9 mg/l) as compared to control (0.47 ± 0.03 mg/l), whereas elicitation of cell suspension culture by MJ and arachidonic acid did not result in biosynthesis of withaferin A. Baldi et al. (2008) later put forth a dual elicitation strategy wherein two different elicitors together could be used together to enhance the biosynthesis of withaferin A. They successfully used *Agrobacterium tumefaciens*-mediated transformation technique for establishing high-yielding callus of Ashwagandha. Different biotic (cell extracts and culture filtrates of *Fusarium solani*, *Alternaria alternata* and *Verticillium dahliae*) and abiotic elicitors (arachidonic acid, calcium chloride, MJ and copper sulphate) were used to elevate the synthesis of withaferin A in the cell suspension culture. Copper sulphate and *V. dahliae* were able to enhance the production to an extent of 5.4 and 9.7 times, respectively, when added solely to suspension cultures as compared to the control (2.65 mg/l). The combination of the two elicitors, that is, dual elicitation method, increased the yield by 13.8-fold.

Chitturi et al. (2010) used fungal culture filtrate and cell extracts of *F. solani* MTCC 350, *A. alternata* MTCC 1779 and *V. dahliae* MTCC 2063 and the abiotic elicitors, viz. calcium chloride, copper sulphate and cinnamic acid, to the suspension cultures of Ashwagandha. They also fed the cell suspension culture with different concentrations of precursors of withanolide biosynthesis like sodium acetate, mevalonolactone, squalene in alcohol, squalene in colloidal form, cholesterol in alcohol and cholesterol in colloidal form for the improvement in the yield of withanolides. Precursors like sodium acetate (tenfold), mevalonolactone (14-fold), squalene in colloidal form (23-fold) and cholesterol in colloidal form (30.5-fold) and elicitors like cells extract of *V. Dahlia* (tenfold) and copper sulphate (2.5-fold) increased the bioproduction of withaferin A. The bioproduction of withaferin A was maximum when precursors and elicitors were added to 3-day-old suspension cultures. Sivanandhan et al. (2012) observed superior production of some major and minor withanolides, like withaferin A, withanolide A, withanone, withanolide B, 12-deoxywithastramonolide, withanolide V and withanoside IV in the adventitious root cultures, when exposed to the biotic elicitors like MJ and salicyclic acid (SA). They optimized the concentration of elicitor, growth index, time of contact with the elicitor as well as the age of adventitious roots for elicitation treatment to enhance withanolides biosynthesis in the Ashwagandha.

The withanolide content increased significantly in the 40 days old culture when the adventitious root cultures were subcultured to media containing150 μM SA with 4 h exposure period on the 30th day, as compared to untreated cultures. The major withanolides were increased considerably: 17.47 mg g/l DW of withaferin A (20-fold), 42.88 mg g/l DW of withanone (37-fold), 33.74 mg g/l DW of withanolide B and 64.65 mg g/l DW of withanolide A (48-fold increase). Recently Sivanandhan et al. (2014a) successfully enhanced the withanolides content and biomass in shoot

suspension culture of Ashwagandha by using the extract of *Gracilaria edulis* and *Sargassum wightii.* They discovered that the addition of the extract of *G. edulis* enhanced the synthesis of the withanolides and biomass by nearly 1.45–1.58-fold as compared to the control culture. Further optimization showed that by exposing the culture with 40% *G. edulis* extract for 24 h in the culture leads to the maximum accumulation of biomass (62.4 g FW and 17.82 g DW) and different withanolides after 5 weeks. This increase in the withanolides was accompanied with increased expression of the enzymes like SE, SS, HMGR and FPS. Sivanandhan et al. (2014b) also studied the influence of addition of precursor molecules and elicitors to cell suspension culture maintained in bioreactor and shake flask, in order to achieve an increased production of Ashwagandha. They used elicitors, namely, cadmium chloride, precursors mevalonic acid and squalene and aluminium chloride and chitosan. Maximum amount of withanolide A, withanolide B, withanone, withaferin A, 12 deoxy withanstramonolide, withanoside IV and V was obtained with combination of chitosan and squalene with KN (0.5 mg/l), picloram (1 mg/L), L-glutamine (200 mg/L) and sucrose (5%) upon culturing for 28 days. The concentration of total withanolides showed 2.13- and 1.66-fold increase in shake-flask culture and bioreactor, respectively. This protocol can be employed on suspension culture for the production of higher yield of withanolides in a short culture period using industrial bioreactors (Sivanandhan et al. 2014b). An increased withanolides production was observed in the hairy root culture of Ashwagandha found that addition of biotic elicitors like *G. edulis* and *S. wightii* extracts and abiotic elicitors such as SA and MJ (Sivanandhan et al. 2013). Kannan and Kulandaivelu (2011) in their experiment on 1-month-old Ashwagandha seedlings in pots found that drought or water stress resulted in an increase of withaferin A by 5%. Various growth characteristic such as root and shoot lengths, leaf area, photosynthetic activity and photosynthetic pigments suffered significant reduction. They proposed that these changes could be due to the variation in enzymatic activity occurring under stress conditions which in turn lead to the production of different compounds through different pathways. These compounds could be different proteins and bioactive compounds which help Ashwagandha tolerate mild drought stress.

1.4.3 Genetic Engineering

Ray and Jha (1999) transformed wild strains of *A. tumefaciens* to the culture of Ashwagandha. Some of the galls infected with N2/73 nopaline strain induced spontaneous teratomas in shoots with abnormal morphology, and these teratomas synthetized higher amount of withanolide D and withaferin A than the non-transformed shoot cultures. Joshi and Dahake (2009) initiated cell suspension cultures of Ashwagandha by transforming the hypocotyls explant with MTCC-2250 strain of *A. tumefaciens*. The cell suspension culture produced withanolides, out of which they isolated withaferin A in the form of white crystals by column chromatography using chloroform-methanol (97:3). Pandey et al. (2010) attempted