Critical factors influencing in vitro propagation and modulation of important secondary metabolites in *Withania somnifera* (L.) dunal

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Abstract

*Withania somnifera* (L.) Dunal is a valuable medicinal plant in the Solanaceae family. It is commonly known as Ashwagandha and is widely distributed around the globe. It has multiple pharmacological properties owing to the existence of diverse secondary metabolites viz., withanolide A, withanolide D, withaferin A, and withanone. It is in great demand in the herbal industry because of its extensive use. In this background, the major challenge lies in the rapid multiplication of elite cultivars of *W. somnifera* in order to produce genetically and phytoconstituents uniform plant material for pharmaceutical industries. Thus it is necessary to explore various biotechnological approaches for the clonal mass propagation and synthesis of pharmaceutically important constituents in *W. somnifera*. Though there are several studies on in vitro propagation on *W. somnifera*, yet many factors that critically influence the in vitro response and withanolides production need to be fine-tuned in the pretext of the existing knowledge. The current review focuses on the advancements and prospects in biotechnological interventions to meet the worldwide demands for *W. somnifera* and its bioactive compounds. This update on in vitro studies on *W. somnifera* will be useful to many researchers, entrepreneurs, and herbal industries looking for its in vitro mass multiplication and scientific utilization.

Key message

*Withania somnifera* is a high value medicinal plant. The present review discusses the advancements in in vitro propagation and important secondary metabolites biosynthesis of *W. somnifera*.

Keywords

Elicitation · In vitro propagation · Meta-Topolin · Cell, Tissue and Organ culture · *Withania somnifera* · Withanolides

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ½ MS         | Half-strength Murashige and Skoog (1962) medium |
| 2,4-D        | 2,4-Dichlorophenoxyacetic acid |
| B5           | Gamborg’s B5 medium |
| BAP          | 6-Benzylaminopurine |
| IAA          | Indole-3-acetic acid |
| IBA          | Indole-3-butyric acid |
| GA3          | Gibberellic acid |
| KN           | Kinetin |
| MS           | Murashige and Skoog (1962) medium |
| mT           | Meta-Topolin |
| NAA          | α-Naphthalene acetic acid |
| PGRs         | Plant growth regulators |
| PP           | Photoperiod |
| RH           | Relative humidity |
| TDZ          | Thidiazuron |
| ZnSO4        | Zinc sulphate |

Introduction

The use of plants as a source of medicine has been depicted in our ancient literature (Dar et al. 2017). There has recently been a resurgence of interest in the usage of therapeutic plants and their secondary metabolites due to their easy availability, low cost, good efficacy, and minimum side effects (Jamshidi-Kia et al. 2018). In addition, more and more experimental evidence gathered on their precise role...
and mode of action against different diseases has lead to their increased acceptability and popularization. According to WHO, it is reported that 80% of the world’s population depends on herbal medicine to treat a variety of health problems (Gupta et al. 2020; Uritu et al. 2018). The trade of medicinal and aromatic plants (MAP) in India is valued at around US$ 238 million, with an annual growth rate of 8% to 10%, while the global market is worth approximately US$ 2.96 billion with a 15–25% annual growth rate (Chandra and Sharma 2019). Further, it is expected that the international trade of herbal products to grow up to $7 trillion by 2050 (Kaur et al. 2021b). The global shift to plant-based medicines, their extraordinary demands, and the associated trades provides an enormous future opportunity to develop strategies for their mass cultivation.

Among medicinal plants, Withania somnifera occupies a distinct position owing to its immense medicinal properties including anti-cancer, cardioprotective, anti-inflammatory, immunomodulatory, anti-coagulant, anti-diabetic, anti-oxidant, and neuroprotective (Kaur et al. 2021a; Sangwan and Sangwan 2014). In silico studies on W. somnifera revealed its potential against COVID-19 by inhibiting the cell surface receptor protein (TMPRSS2) and viral protein (Mpro) of SARS-CoV-2 (Dhanjal et al. 2021). The potent therapeutic potential of this plant is mainly due to the existence of different bioactive compounds including alkaloids, steroidal lactones, glycowithanolides, sterols, flavonoids, and phenolics (Chaurasiya et al. 2012). Among these, steroidal lactones viz., withanolide A, withanolide D, withanolide E-M, withaferin A, and withanine have attracted the attention of researchers due to their wide range of pharmacological properties (Namdeo and Ingawale 2020; Sivanandhan et al. 2020). These secondary metabolites are differentially distributed in various parts of W. somnifera like leaves, fruits, and roots (Namdeo et al. 2011). Withanone and withaferin A were detected in leaves, while withanolide A, sitoindoside VIII, and sitoindoside VIII were found in roots (Karthikeyan et al. 2019; Singh et al. 2015). Further, MS and NMR spectroscopy studies on fruits of W. somnifera identified 82 chemically diverse metabolites including withanamides, phenolic acids, sterols, and tocopherols (Bhatia et al. 2013). This plant has been found to contain more than 12 alkaloids, 40 withanolides, and several sitoindosides (Mir et al. 2012). At present, W. somnifera is utilized in various herbal medicines, resulting in high demand in a global marketplace.

Due to the large demand of W. somnifera, it is being cultivated in different states of India including Jammu and Kashmir, Himachal Pradesh, Maharashtra, Punjab, Rajasthan, Karnataka, Andhra Pradesh, Madhya Pradesh, Tamil Nadu, Uttar Pradesh, and Gujarat (Kulkarni and Dhir 2008; Kumar et al. 2007; Lal 2015; Meher et al. 2016). In India, the annual production of W. somnifera is estimated to be 1500 tonnes, while annual consumption is anticipated to be 7000 tonnes resulting in a substantial demand-supply gap (Kaur et al. 2021b). In addition, W. somnifera which is normally propagated by seeds suffers limitations due to the low seed viability, low seed germination percentage, and less seedlings survival rate (Kaur et al. 2021b). Further, the cultivation and its genetic improvement processes are affected owing to its narrow genetic base, self-pollination, long gestation period, and infestation by various pathogens and insects (Singh et al. 2017). All these factors suggest that the conventional methods of propagation of W. somnifera and its genetic improvement to meet the commercial demand are difficult to accomplish. Hence, deciphering strategies for their rapid propagation using modern experimental techniques could be beneficial.

Since many years, in vitro propagation through plant tissue culture techniques has proved itself as a reliable and promising tool for clonal propagation of healthy and disease-free plants throughout the year. In the case of W. somnifera, this technology could be of immense value as it facilitates in generating large-scale healthy, genetically uniform plants with defined chemical content for pre-clinical and translational studies. Overall, the rapid propagation of elite plants will provide high dividends to farmers and the associated herbal industry. In addition, the integration of plant tissue culture with other biotechnological tools can be employed to enhance the commercial value of W. somnifera. Thus, the present review provides a summarized view of attempts undertaken for plant cell, tissue and organ culture in W. somnifera. It also highlights the experimental strategies adapted to increase the biosynthesis of pharmacologically important constituents in this high valued medicinal plant.

### In vitro propagation in W. somnifera

#### Micropropagation

In recent years, several efforts have been undertaken to propagate W. somnifera employing different explants (Kaur et al. 2017; Singh et al. 2017). The successful micropropagation protocol of W. somnifera involves several distinct and interrelated stages. These include (i) initiation of aseptic cultures, (ii) shoot multiplication, (iii) in vitro rooting, (iv) acclimatization of rooted microshoots, and further establishment in field conditions (Fig. 1). Herein, an attempt is made to discuss the current status of work on micropropagation of W. somnifera and various factors which greatly influence the different stages of micropropagation.

#### Initiation of aseptic cultures

Initiation of aseptic culture is the first and critical stage in successful micropropagation protocol. It comprises explant selection and sterilization.
Choice of explant

The selection of explants largely depends on the objective of the experiment. In the case of *W. somnifera*, different explants like nodal segments, seeds, and shoot tips were used to initiate aseptic cultures (Table 1). The majority of studies in *W. somnifera* have used nodal segments for the establishment of in vitro cultures.

Surface sterilization of explants

Several factors including type, concentration, and treatment duration of the sterilizing agents influence the surface sterilization of explants (Wen et al. 2020). The surface sterilization of *W. somnifera* is often accomplished by the use of various sterilizing agents in a step-wise manner. Presterilization of the explants is conducted by washing of explants with distilled water followed by treatment with some commercial detergents (Table 1). Teepol was employed as a detergent at a concentration of 5% (v/v) for 5–8 min (Nayak et al. 2013; Ray and Jha 2001; Sen and Sharma 1991), although other researchers specified 2.5% (v/v) teepol for 5 min (Sivanandhan et al. 2011, 2015). Labolene (5%) has also been used for a longer time (10–15 min) for surface sterilization of explants (Fatima et al. 2011; Fatima and Anis 2011, 2012). Besides these, the lower percentage of Tween-20 (1%) for 1 min was used for the sterilization of nodal segments (Autade et al. 2016) whereas, a higher percentage (10%) of Tween-20 was also reported for the sterilization of axillary buds in *W. somnifera* for 5 min (Saritha and Naidu 2007). For surface sterilization of explants, various sterilizing agents such as mercuric chloride (HgCl₂) and sodium hypochlorite (NaOCl) were used in *W. somnifera* (Table 1). Several workers have used different doses of HgCl₂ solutions such as 0.01%, 0.1%, and 0.2% (w/v) for sterilization in *W. somnifera* (Autade et al. 2016; Kannan and Anbazhakan 2016; Sen and Sharma 1991; Sivanandhan et al. 2011; Supe et al. 2006). The sterilization of explants with 0.4% NaOCl has also been documented in *W. somnifera* (Kaur et al. 2021b; Udayakumar et al. 2013).

Critical factors influencing micropropagation

Medium

Various types of basal media were employed for experiment-specific purposes in micropropagation of *W. somnifera* and the most frequently used nutrient media is Murashige and Skoog (Murashige and Skoog 1962) medium (Kaur et al. 2017; Singh et al. 2017) (Table 1). In addition, a large number of studies in micropropagation of *W. somnifera* have reported various other types of basal media such as B5 media, Carbon/nitrogen sources, PGRs, Additives, Culture conditions, Elicitors, and Precursor feeding

In vitro shoots

Direct adventitious shoot organogenesis
Callus mediated shoot organogenesis

In vitro nodal explants

Multi-step subculture protocol

In vitro shoots

Direct adventitious shoot organogenesis
Callus mediated shoot organogenesis

In vitro shoots

Critical factors influencing micropropagation

Medium

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### Table 1  Critical factors influencing micropropagation in *W. somnifera*

| Explant | Stages of micropropagation | Reference |
|---------|---------------------------|-----------|
|         | Aseptic cultures (Sterilization process) | Shoot multiplication (Media + PGRs + other additives) | Rooting (Media + PGRs) | Hardening (Potting mixture) |
| Seeds   | 5% Teepol solution (5 min); 0.1% HgCl₂ (12–13 min) | MS + BAP (4.4 µM) | MS liquid | Soil | (Sen and Sharma 1991) |
|         | 0.1% (w/v) HgCl₂ (5 min) | MS + BAP (4.4 µM) + 2,4-D (2.3 µM) | ↓ | | |
|         | 0.2% (v/v) Tween-20; 90% Ethyl Alcohol (10–15 s); 0.1% HgCl₂ (2 min) | MS + BAP (0.6 mg/l) + IAA (0.4 mg/l) | MS + IAA (0.4 mg/l) + IBA (0.4 mg/l) | Sand: Soil (1: 1) | (Supe et al. 2006) |
| Nodal explant from field growing plants | Teepol (5 min); 70% (v/v) ethanol (1 min) 0.2% HgCl₂ (5 min) | MS + BAP (1.5 mg/l) + IAA (1.5 mg/l) | ½ MS + IBA (2.0 mg/l) | Sand: Soil: vermiculite (1:1:1) | (Sivanesan and Murugesan 2008) |
|         | 5% (v/v) Teepol (5 min); 0.1% (w/v) HgCl₂ (10 min); 70% Alcohol | MS + BAP (1.0 mg/l) + KN (1.0 mg/l) | MS + IBA (2.0 mg/l) | Garden soil: Vermi-compost (3:1) | (Sabir et al. 2008) |
|         | 2.5% (v/v) Teepol (5 min); 0.1% HgCl₂ (2 min); Ethanol (2 min) | MS + BAP (1.5 mg/l) + IAA (0.3 mg/l) + Spermidine (20 mg/l) | MS + Putrescine (20 mg/l) | Sand: Soil: Vermiculite (1:1:1) | (Sivanandhan et al. 2011) |
|         | 2.5% (v/v) Teepol (5 min); 0.1% (w/v) HgCl₂ | MS + BAP (1.5 mg/l) + IAA (0.3 mg/l) + Glutamine (20 mg/l) | MS + IBA (2.0 mg/l) + AN (15 mg/l) | - | (Sivanandhan et al. 2015) |
| Cotyledonal nodes | 5% (v/v) Labolene (5 min); 0.1% (w/v) HgCl₂ (4 min) | MS + BAP (2.5 µM) + NAA (0.5 µM) | MS + NAA (200 µM); 30 min | Soilrite | (Fatima et al. 2015) |
|         | 1% Tween-20 (1 min); 70% Ethanol (1 min); 0.1% HgCl₂ (7–8 min) | MS + BAP (0.5 mg/l) + NAA (1.5 mg/l) | MS + IBA (2.0 mg/l) | Soil: Sand: Vermiculite (1:2:1) | (Autade et al. 2016) |
|         | 0.4% (v/v) NaOCl; 0.1% (w/v) HgCl₂ (3 min) | MS + BAP (2.5 µM) | 1/6 AN-MS | Soil: Sand: Vermiculite (1:1:1) | (Kaur et al. 2021b) |
| Shoot tips from field growing plants | 5% (v/v) Teepol (8 min); 0.1% (w/v) HgCl₂ (5 min) | MS + BAP (1.0 mg/l) | ½ MS + IBA (1.0 mg/l) | Soil: Sand: Compost (1:1:1) | (Nayak et al. 2013) |
|         | 5% Teepol (5 min); 0.1% HgCl₂ (20 min) | MS + BAP (1.0 mg/l) | ½ MS + IBA (2.0 mg/l) | - | (Ray and Jha 2001) |
|         | Teepol (10 min); 70% Ethanol (1 min); 0.2% HgCl₂ (5 min) | MS + BAP (2.0 mg/l) + IAA (2.0 mg/l) | ½ MS + IBA (2.0 mg/l) | - | (Sivanesan 2007) |
### Table 1 (continued)

| Explant                           | Stages of micropropagation | Reference                |
|-----------------------------------|----------------------------|--------------------------|
|                                   | Aseptic cultures (Sterilization process) |                         |
|                                   | Shoot multiplication (Media + PGRs + other additives) |                         |
|                                   | Rooting (Media + PGRs) |                         |
|                                   | Hardening (Potting mixture) |                         |
| Axillary buds                     | 1% mild detergent (2–3 min); 0.1% HgCl₂ (1–2 min) | MS + BAP (0.25 mg/l) | MS + NAA (0.5 mg/l) | Soil: Sand (3:1) | (Sharma et al. 2009) |
|                                   | 1% Lavelone (30 min); 0.1% HgCl₂; 70% Ethanol (5 s) | MS + KN (0.5 mg/l) + BAP (1.0 mg/l) | MS | - | (Sangwan et al. 2008) |
| Nodal explant from in vitro seedlings | 5% (v/v) Lavelone (10 min); 0.1% (w/v) HgCl₂ (4 min) | MS + BAP (2.5 mM) + NAA (0.5 mM) | ½ MS + NAA (0.5 mM) | Garden soil: Vermicompost (3:1) | (Fatima and Anis 2012) |
|                                   | 5% (v/v) Lavelone (15 min); 0.1% (w/v) HgCl₂ (4 min) | MS + BAP (2.5 mM) + NAA (0.5 mM) + ZnSO₄ (300 μM) | ½ MS + NAA (0.5 mM) | Soilrite | (Fatima et al. 2011) |
| Shoot tips from in vitro seedlings | - | NN + BA (1.0 mg/l) + IBA (1.0 mg/l) | NN + KN (0.1 mg/l) + IBA (0.5 mg/l) + ADS (10.0 mg/l) | Sand and Soil | (Furmanowa et al. 2001) |
|                                   | 5% Teepol (5 min); 0.1% HgCl₂ (12–13 min) | MS + BAP (4.4 μM) + IBA (2.5 μM) | MS liquid | Soil | (Sen and Sharma 1991) |

*MS*: Murashige and Skoog (1962) medium; ½ MS: half-strength Murashige and Skoog (1962) medium; 1/6 AN-MS: 1/6 strength of ammonium nitrate in MS medium; BAP: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; IBA: indole-3-butyric acid; PGRs: plant growth regulators; NAA: α-naphthalene acetic acid; KN: kinetin; GA: gibberellic acid; NN: Nitsch and Nitsch (1969) medium; ZnSO₄: zinc sulphate; ADS: adenine sulphate

*Mostly all these reports used MS medium with 3% sucrose and agar as a gelling agent*
(Gamborg et al. 1968) and Schenk and Hildebrandt (SH) (Schenk and Hildebrandt 1972), Nitsch and Nitsch (NN) (Nitsch and Nitsch 1969), and woody plant medium (WPM) (Lloyd and McCown 1980) by different workers (Fatima et al. 2015; Furmanowa et al. 2001; Namdeo and Ingawale 2020; Shasmita et al. 2018; Sivanesan 2007). MS medium containing plant growth regulators (PGRs) showed the maximum percentage of shoot induction from apical buds in comparison to B5 and SH medium (Sivanesan 2007). This could be due to the moderate salt requirement for shoot proliferation of W. somnifera. Furthermore, MS medium was reported to be optimal for shoot multiplication among B5, MS, and WPM medium from nodal segments (Fatima et al. 2015). The improved growth of W. somnifera shoots in MS medium could be due to the higher concentration of ammonium and nitrate in comparison to WPM and B5 medium (Ahmad and Anis 2011; Perveen et al. 2011). The use of NN medium enriched with 1.0 mg/l BA (6-benzylaminopurine) and 1.0 mg/l IBA (indole-3-butyric acid) was also used for shoot multiplication (Furmanowa et al. 2001).

The role of different strengths MS medium was also investigated for root induction (Table 1). In certain cases, a half-strength MS (1/2 MS) medium was adopted for in vitro rooting of W. somnifera shoots (Fatima et al. 2011; Fatima and Anis 2012; Kannan and Anbazhakan 2016; Nayak et al. 2013; Saema et al. 2015; Sivanesan 2007). It was observed that a higher percentage of cultures were responded to rooting on 1/2 MS medium (42.5%) in comparison with full-strength MS medium when shoots were cultured on the same concentration of auxin i.e. IBA (20.0 µM) (Sharma et al. 2015). The higher frequency (100%) of rooting with the maximum number of roots was also reported on 1/2 MS medium containing IBA (2.0 mg/l) and 1.5% sucrose (Sivanesan and Park 2015). It is well known that the culture medium containing a higher level of nutrients with high osmotic potential showed inhibition to root induction (George et al. 2008). This might be the reason for the higher rate of in vitro rooting in the reduced strength of the culture medium. Our research group revealed that 1/6 strength of NH$_4$NO$_3$ in basal MS medium favors in vitro rooting compared with full-strength MS (Kaur et al. 2021b). This might be due to the fact that nitrogen affects auxin biosynthesis, signaling, and transport in plants (Vega et al. 2019). On contrary, the higher rate of root induction in W. somnifera was also reported in full-strength MS medium containing IBA (1.0 mg/l) in comparison to the reduced strengths (1/2 MS, 1/3 MS, and 1/4 MS) of basal MS medium (Ghimire et al. 2010).

Furthermore, the addition of additives like L-glutamine (Sivanandhan et al. 2015), spermidine (Sivanandhan et al. 2011), ZnSO$_4$ (Fatima et al. 2011), and coconut milk (Ray and Jha 2001) to the nutrient medium improved shoot multiplication in W. somnifera. Moreover, the role of various polyamines such as putrescine, spermine, and spermidine in in vitro rooting was also investigated (Sivanandhan et al. 2011). Among these, putrescine (20 mg/l) was found effective for in vitro rooting as compared with spermine and spermidine. The addition of 10.0 mg/l adenine sulphate (Furmanowa et al. 2001) and 15.0 mg/l ammonium nitrate (Sivanandhan et al. 2015) in IBA supplemented culture medium also influenced the root induction.

Growth regulators

Growth regulators free basal culture medium is not sufficient for in vitro multiple shoot proliferation. In vitro shoot multiplication is possible with the addition of PGRs in the basal culture medium. In W. somnifera, BAP was favorably used for optimum shoot proliferation either alone (Kannan and Anbazhakan 2016; Nayak et al. 2013; Saema et al. 2015; Sharma et al. 2015, 2009) or in conjunction with different auxins such as indole-3-acetic acid (IAA) (Sivanandhan et al. 2011, 2015; Sivanesan 2007; Sivanesan and Murugesan 2008), IBA (Furmanowa et al. 2001; Sen and Sharma 1991), and α-naphthalene acetic acid (NAA) (Fatima et al. 2011; Fatima and Anis 2012; Saritha and Naidu 2007) (Table 1). Furthermore, different types of cytokinins such as kinetin (KN) and thidiazuron (TDZ) were studied for shoot proliferation in W. somnifera (Fatima and Anis 2011; Sangwan et al. 2008; Sivanesan and Park 2015). BAP is most commonly used for shoot multiplication in W. somnifera (Singh et al. 2017) due to high cytokinin activity, accessibility, and relatively inexpensive. Kinetin is a weak cytokinin that promotes shoot multiplication with the addition of BAP in W. somnifera (Deka et al. 1999; Mir et al. 2014; Sabir et al. 2008; Sangwan et al. 2008). On the other hand, TDZ (thidiazuron), a substituted phenylurea is an effective cytokinin at very low concentrations for shoot multiplication (Fatima and Anis 2011). This TDZ action is attributed to an intrinsic cytokinin-like activity that inhibits cytokinin oxidases (Mohammadi et al. 2020). Unlike other Solanaceaous species, W. somnifera plants are not easy to establish long-term under in vitro conditions. Kaur et al. (2021b) recorded that the repeated culturing of W. somnifera shoots in MS medium supplemented with BAP showed a sharp decline in the shoot multiplication index. In addition, prolonged BAP exposure caused morphological changes including leaf yellowing and necrosis, stunted shoot growth, thick stem, and hyperhydricity. The negative influence of BAP could be ascribed to its high stability, which causes an increase in BAP concentration in the microshoots (Kaur et al. 2021b). To overcome this problem, a multistep subculture protocol comprising auxin and cytokinin has been employed. But the rate of shoot multiplication did not increase significantly with each subculture. As a result, it was necessary to fine-tune such protocols using an unconventional cytokinin to improve
plant multiplication and growth. Recently, our group has investigated the use of novel cytokinin, i.e. meta-Topolin (mT), BAP analog for shoot multiplication in W. somnifera (Kaur et al. 2021a). mT (6-(3-hydroxybenzylamino) purine) is an aromatic natural cytokinin and isolated from poplar leaves (Chauhan and Taylor, 2018; Strnad et al., 1997). The effective shoot multiplication with the application of mT has been reported in various plant species (Behera et al. 2019, 2022; Halder and Ghosh 2021; Kucharska et al. 2020; Naaz et al. 2019) and it also showed a higher efficacy over BAP for shoot multiplication in W. somnifera (Kaur et al. 2021a). This could be because the aromatic side chain has a hydroxyl group, which facilitates the formation of O-glycoside. Depending on the requirements, these O-glycosides can be easily converted into active nucleotides, nucleosides, or free bases (Shekhawat et al. 2021). For the elongation of proliferated microshoots, the use of gibberellic acid (GA3) supplemented MS medium has been recorded in W. somnifera (Sivanandhan and Murugesan 2008; Sivanesan and Park 2015).

MS media supplemented with different auxins such as IAA, IBA, and NAA alone or in conjunction with each other was employed for in vitro rooting of microshoots in W. somnifera (Table 1). Auxin promotes lateral root initiation and primordium growth by stimulating cell division, expansion, and differentiation (Kaur et al. 2021b). In previous reports, IBA was favorably used for root induction in W. somnifera (Singh et al., 2017; Shasmita et al. 2018). Besides this, IBA in conjunction with other auxins such as IAA (Rani and Grover 1999; Supe et al. 2006) and NAA (Arumugam and Gopinath 2013; Fatima et al. 2011; Fatima and Anis 2012; Sharma et al. 2009) has also been reported for in vitro rooting.

### Carbon sources

Carbon is essential for the growth and development of in vitro grown cultures of many plant species. It is provided in the form of sugars including glucose, maltose, sucrose, and fructose. They serve as energy sources and keep the osmotic balance between both the cell and the external environment in the growth medium (George et al. 2008; Sivanesan and Park 2015). Sucrose is commonly used as a carbohydrate source due to its easy translocatability and resistance to enzymatic degradation (Fatima et al. 2015). Different concentrations (1%, 2%, 3%, 4%, 6%, and 8%) of sucrose were reported in in vitro propagation studies of W. somnifera depending upon the objective of the experiment. 1% sucrose was used for seed germination (Sen and Sharma 1991; Supe et al. 2006) while 3% sucrose was used for shoot multiplication through meristem culture (Fatima and Anis 2011, 2012; Saema et al. 2015; Sivanesan and Murugesan 2008). Whereas, the higher concentration (4%) of sucrose was also reported for shoot multiplication from nodal segments (Sivanandhan et al. 2015). Besides the shoot proliferation, the higher concentration (6%) of sucrose in MS medium along with BA (0.3 mg/l) showed in vitro flowering in W. somnifera (Sivanesan and Park 2015) and the same effect of sucrose was also documented in many other plant species (Franklin et al. 2000; Rathore et al. 2013; Sangee-tha and Venkatakalam 2014). On the other hand, a lower concentration of sucrose was preferred for in vitro rooting of W. somnifera (Sivanesan and Park 2015). In this report, the maximum root induction was achieved with 1.5% sucrose over 3% sucrose in IBA (2.0 mg/l). This study clues that there is some kind of connection between sucrose concentration and root-promoting PGR i.e. auxin. Sucrose is hydrolyzed into glucose and fructose in the nutrient medium and glucose can regulate auxin biosynthetic genes (YUCCA), transporter (PIN proteins), receptor (TIR1), and genes involved in auxin signaling (GH3, AUX/IAA, and SAUR) resulting in the enhancement in root induction (Mishra et al. 2009).

### Status of the medium

Agar, phytagel, clarigel, and gelrite are used as gelling agents to prepare a semi-solid culture medium (Kaur and Kumar 2020). In the case of W. somnifera, agar and phytagel were used as the gelling agents to support explants. Agar, a polysaccharide obtained from seaweeds is most frequently used for in vitro propagation of W. somnifera due to its convenient gelling properties, stability, and resistance to degradation by plant enzymes (Purohit et al. 2011). However, several limitations including batch-to-batch inconsistency, limited availability, presence of impurities, and inhibition of growth have been recorded on the use of agar in the culture medium (Puchhoa et al. 1999). In addition, agar is the most expensive component in plant tissue culture medium and is responsible for around 70–80% of the total cost (Lozzi et al. 2019). On the other hand, phytagel which is a natural polysaccharide has also been used as a gelling agent in shoot proliferation of W. somnifera (Sivanandhan et al. 2011, 2015). To develop the micropropagation protocol for commercialization, the fine-tuning of existing protocols as well as cost reduction is very critical (Pati et al. 2006). The elimination of gelling agents which saves substantial cost has been tried by many researchers in various plant species (Malik et al. 2016; Singh 2018; Vaidya et al. 2019; Wangdi and Sarethy 2016). The liquid culture system has a number of potential benefits including (i) improved plant growth due to increased availability of nutrients and growth regulators, (ii) adequate aeration in a liquid medium which results in the improvement of shoot growth and multiplication, (iii) sterilization by microfiltration, (iv) addition of fresh media without changing the container, and (v) high efficiency of transferring plants to their natural conditions (Lozzi et al. 2019).
recorded when the soil was mixed with sand to acclimatize Whereas, the higher percentage survival (83–100%) was gated plants were transferred to soil (Sen and Sharma 1991). The age of survival (20%) was achieved when in vitro propagation mixture, light intensity, and humidity conditions of the greenhouse (da Silva et al. 2017). Different research groups on various factors such as the genotype of the plant, potting mixture, light intensity, and humidity conditions of the tissue culture room lies between the range of 35–45 μmol/m²/sec (Singh et al. 2017). The higher (60 μmol/m²/sec) light intensity was also reported for multiple shoot induction (Saema et al. 2015). In the majority of the experiments, 16 h light/8 h dark conditions were most commonly executed. However, 12 h light/12 h dark and 20 h light/4 h dark conditions were also reported for shoot multiplication (Furmanowa et al. 2001; Saritha and Naidu 2007). Further, the relative humidity (RH) in the tissue culture room was kept between 50–70% in W. somnifera (Kaur et al. 2021b; Saritha and Naidu 2007; Sivanandhan et al. 2015).

Culture conditions

For effective in vitro propagation, the microenvironment of the tissue culture room viz., temperature, light intensity, photoperiod, and relative humidity (RH) is essential to maintain cell growth. In the majority of reports published in W. somnifera, the temperature range is similar and lies between the ranges of 20–25 ± 1–2°C (Kaur et al. 2021b; Sangwan et al. 2008; Sivanandhan et al. 2013). The optimum condition of light intensity of the culture room is imperative for better biomass production as light is a primary factor for photosynthesis in plants. In W. somnifera, the light intensity of the tissue culture room lies between the range of 35–45 μmol/m²/sec. However, 12 h light/12 h dark and 20 h light/4 h dark conditions were also reported for shoot multiplication (Furmanowa et al. 2001; Saritha and Naidu 2007). Further, the relative humidity (RH) in the tissue culture room was kept between 50–70% in W. somnifera (Kaur et al. 2021b; Saritha and Naidu 2007; Sivanandhan et al. 2015).

Hardening and field establishment

Hardening is the last step of in vitro propagation study. Successful acclimatization of in vitro raised plants depends on various factors such as the genotype of the plant, potting mixture, light intensity, and humidity conditions of the greenhouse (da Silva et al. 2017). Different research groups working on W. somnifera employed different approaches to harden tissue culture-raised plants. Many different kinds of potting mixes either individually or in combination were used to acclimatize W. somnifera (Table 1). A lower percentage of survival (20%) was achieved when in vitro propagated plants were transferred to soil (Sen and Sharma 1991). Whereas, the higher percentage survival (83–100%) was recorded when the soil was mixed with sand to acclimatize tissue culture raised plants (Furmanowa et al. 2001; Kulkarni et al. 1996, 2000; Rani and Grover 1999; Sharma et al. 2015, 2009; Supe et al. 2006). This might be attributed to the sand’s high porosity and rate of percolation. Further, a high survival rate was also recorded on soilrite, soil: sand: vermiculite, garden soil: vermicompost, sand: soil: compost, soil: leaf manure, soil:ite: soil: compost, vermiculite: perlite, cow dung: red soil, garden soil: sand: farmyard manure, river sand: soil: vermicompost, and agropeat (Shasmita et al. 2018). Overall, these reports suggested that the potting mixture containing soil, sand, and vermiculite facilitated 100% successful acclimatization of micropropagated plants.

Clonal fidelity assessment of micropropagated shoots

Various approaches such as molecular markers, morphophysiological, cytological, and biochemical assays were used to evaluate the genetic fidelity of in vitro propagated plants (Gantait et al. 2015). Among these, molecular markers are the method of choice because they are simple, quick, and only require a small quantity of plant material (Kaur et al. 2021b). There are a large number of reports available on micropropagation of W. somnifera. However, the genetic fidelity of regenerants is carried out in a few reports only (Fatima et al. 2015; Kaur et al. 2021b; Mallubhotla et al. 2008; Nayak et al. 2013). To evaluate the genetic uniformity of nodal explant-based in vitro propagated plants in W. somnifera, OPA 04, OPA 09, HBV5, and HVR were selected based on banding pattern out of 20 RAPD (Random amplified polymorphic DNA) and 3 DAMD (Directed amplification of minisatellite DNA) primers (Fatima et al. 2015). The banding pattern profiles of all the micropropagated plants and mother plant were monomorphic. Further, the clonal fidelity of in vitro propagated plants from cotyledonary nodes of W. somnifera was also carried out using ISSR (Inter simple sequence repeats) and RAPD markers (Nayak et al. 2013). Amplified products generated from primers showed homogeneous bands, thus validating the true-to-type nature of in vitro propagated plants. Furthermore, three different PCR-based molecular markers including ISSR, RAPD, and SCoT (Start codon targeted) were employed to access the clonal fidelity of in vitro grown plants of W. somnifera for 3 consecutive years (Kaur et al. 2021b).

In vitro regeneration in W. somnifera

An effective and reproducible regeneration system is required for the implementation of biotechnological techniques used for plant improvement programs. The addition of PGRs in the culture media is the most important factor for organogenesis. However, the type and concentration of
growth regulators added to the nutrient medium determine whether an explant regenerates by direct or indirect mode of shoot organogenesis.

Unlike other solanaceous species (Nicotiana, Petunia, and Datura), Withania is a highly recalcitrant species with regard to its capacity for in vitro plant regeneration and genetic transformation (Bomzan et al. 2020; Yildiz 2012). It is well known that the plants that produce high levels of secondary metabolites are recalcitrant to plant regeneration (Benson 2000; Pandey et al. 2010). There are only a few studies on direct shoot regeneration in W. somnifera (Table 2) and leaves were most frequently used as explants (Ghimire et al. 2010; Joshi and Padhya 2010; Kaur et al. 2021a; Kumar et al. 2011; Logesh et al. 2010). Few studies were also available on the use of other explants, such as epicotyl (Udayakumar et al. 2013), petiole (Ghimire et al. 2010), and internodes (Kulkarni et al. 2000) for direct shoot organogenesis. The different types of basal medium (MS, B5, and SH) and carbon sources (sucrose, fructose, and maltose) influence the shoot regeneration frequency from leaf and petiole explants in W. somnifera (Ghimire et al. 2010). They investigated that MS medium with 3% sucrose was optimum for the induction of direct adventitious shoots. Similarly, many other researchers have also reported the use of 3% sucrose for direct shoot regeneration (Joshi and Padhya 2010; Kaur et al. 2021a; Kumar et al. 2011). On contrary, 2% sucrose supplemented MS medium was also recorded for shoot organogenesis (Kulkarni et al. 1996, 2000). Apart from the culture media composition, PGRs are also critical in shoot regeneration. In most of the studies, BAP was most commonly used cytokinin either alone (Ghimire et al. 2010; Kaur et al. 2021a; Kulkarni et al. 2020) or in combination with IAA (Ghimire et al. 2010; Logesh et al. 2010), IBA (Ghimire et al. 2010; Logesh et al. 2010), and KN (Joshi and Padhya 2010; Kaur et al. 2021a; Kumar et al. 2011).

Table 2  Factors influencing direct adventitious shoot organogenesis in W. somnifera

| Explant                              | Direct shoot organogenesis | Rooting (Medium + PGRs) | Reference                  |
|--------------------------------------|---------------------------|-------------------------|----------------------------|
|                                      | Media + PGRs              | Shoot regeneration frequency (%) | No. of shoots/explant | Reference                  |
| Leaves from in vitro seedlings       | MS + BAP (8.8 μM) + IAA (7.99 μM) | 56.65%                  | 16                         | MS + BAP (0.044 μM) (Kulkarni et al. 1996) |
|                                      | MS + BAP (2.0 mg/l)       | –                       | 23                         | MS + IBA (1.0 mg/l) (Ghimire et al. 2010) |
|                                      | MS + BAP (2.0 mg/l) + IAA (0.5 mg/l) | –                       | –                         | MS + IBA (0.5 mg/l) (Logesh et al. 2010) |
|                                      | MS + GA3 (0.5 mg/l)       | ↓                       |                            |                            |
| Leaves from in vitro grown shoots    | MS + BAP (10.0 μM)        | 92.58%                  | 23.13                      | MS + IAA (10.0 μM) (Kaur et al. 2021a) |
| Leaves from field growing plants     | MS + BAP (4.0 μM) + KN (4.0 μM) | 90%                     | 12.1                       | ½ MS + BA (0.5 μM) + Sucrose (1%) (Joshi and Padhya 2010) |
| Internode explants from in vitro seedlings | MS + BAP (5.0 mg/l)       | –                       | 10.22                      | MS + BAP (0.01 mg/l) (Kulkarni et al. 2000) |
|                                      | MS + BAP (0.01 mg/l)      | ↓                       |                            |                            |
|                                      | MS + KN (2.0 mg/l) + NAA (0.5 mg/l) + GA3 (0.3 mg/l) | 96%                     | 22.8                       | ½ MS + IBA (2.0 mg/l) + Sucrose (1.5%) (Sivanesan and Park 2015) |
| Nodal segments from in vitro seedlings | MS + TDZ (0.2 mg/l) + Sucrose (2%) | –                       | 10.1                       | ½ MS (Kulkarni et al. 2000) |
|                                      | ↓                         |                         |                            |                            |
| Epicotyls from in vitro seedlings    | MS + BAP (2.0 mg/l) + IAA (0.2 mg/l) | 85%                     | 6.6                        | MS + IBA (0.8 mg/l) (Udayakumar et al. 2013) |
|                                      | MS + GA3 (1.0 mg/l)       | ↓                       |                            |                            |
| Leaf petiole                         | MS + BAP (2.0 mg/l)       | –                       | 3.67                       | MS + IBA (1.0 mg/l) (Ghimire et al. 2010) |

* Mostly all these reports used MS medium with 3% sucrose and agar as a gelling agent

MS: Murashige and Skoog (1962) medium; PGRs: plant growth regulators; IAA: indole-3-acetic acid; GA3: gibberellic acid; BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid; TDZ: thidiazuron; ½ MS: half-strength Murashige and Skoog (1962) medium; KN: kinetin
et al. 2000) or in conjunction with kinetin (Joshi and Padhya 2010) or IAA (Kulkarni et al. 1996; Kumar et al. 2011; Logesh et al. 2010; Udayakumar et al. 2013) in W. somnifera. In most of the reports, the highest shoot regeneration frequency (> 80%) was obtained on MS medium fortified with BAP alone or in conjunction with auxin and another mild cytokinin irrespective of the explant type (Table 2). These studies revealed the importance of BAP in the shoot regeneration medium for in vitro direct shoot bud induction using various explants in W. somnifera. The types and orientation of the explant were shown to have a substantial impact on the shoot regeneration frequency (Ghimire et al. 2010). They reported the higher shoot regeneration frequency from leaf segments in comparison to petiole explant. This might be owing to the basipetal transport of endogenous auxins. They also investigated that the petioles responded to shoot regeneration from their petiolar region due to higher density of vascular tissue, phytohormones levels, and metabolites (Ghimire et al. 2010). Additionally, the role of culture vessels in plant tissue culture has long been documented. The leaves inoculated in glass culture tubes showed maximum shoot induction. On the other hand, only callus formation was recorded while leaf explants were inoculated on plastic Petri-dishes (Kulkarni et al. 1996). This might be owing to the high amount of ethylene accumulation in glass tubes that could promote the shoot bud induction through controlling cell division in the induction phase (Shasmita et al. 2018).

In Indirect shoot organogenesis of W. somnifera, different explants such as the leaf, cotyledons, epicotyl, hypocotyl, axillary shoots, shoot tips, axillary leaves, and internodes have been used (Table 3). The leaves and nodal explants were commonly employed for indirect shoot organogenesis (Arumugam and Gopinath 2013; Dewir et al. 2010; Rani et al. 2016; Shukla et al. 2010; Waman et al. 2011). Further, MS medium with 3% sucrose and 0.6–0.8% agar was mostly reported for indirect shoot organogenesis. Among the different plant growth regulators, 2,4-D alone or in conjunction with KN was shown to be efficient for inducing callus from different explants in W. somnifera (Shasmita et al. 2018; Singh et al. 2017). Furthermore, the callus-mediated shoot regeneration was recorded in BAP alone or in conjunction with IAA or NAA, or IBA (Shasmita et al. 2018). In few reports, the same composition of callus induction medium has also been used for shoot regeneration (Rani and Grover 1999; Shukla et al. 2010).

Advancements in the production of withanolides

Plant cell, tissue and organ cultures have evolved as promising resources for the production of pharmaceutically important secondary metabolites owing to its advantages over traditional cultivation including a year round-system for the synthesis of pharmaceutically important compounds without any geographical, seasonal, and environmental variations, simplicity in the extraction of metabolites, and high yields, etc. (Gonçalves and Romano 2018; Isah et al. 2018). In the past few years, various approaches have been hypothesized and experimented with to enhance the biosynthesis of withanolides in W. somnifera (Fig. 1). Some of these include tissue and organ culture, hairy root culture, cell suspension culture, elicitation, precursor feeding, large-scale cultivation in a bioreactor system, and biotransformation (Namdeo and Ingawale 2020). In this review, we discuss tissue and organ culture as one of the approaches for increasing withanolides production in W. somnifera.

Critical factors influencing withanolides production in tissue and organ culture

Different factors such as type of explants (Sharada et al. 2007; Sabir et al. 2013; Sivanandhan et al. 2011), media (Praveen and Murthy 2010; Ray and Jha 2001), growth regulators (Adil et al. 2019; Rangaraju et al. 2018; Ray and Jha 2001; Sivanandhan et al. 2012a, 2013), carbon and nitrogen sources (Ray and Jha 2001; Sivanandhan et al. 2015), the status of the medium (Mir et al. 2014; Sivanandhan et al. 2013; Ray and Jha 2001; Senthil et al. 2015; Rangaraju et al. 2019, 2018), additives (Ray and Jha 1991; Sivanandhan et al. 2013), and elicitors (Sivanandhan et al. 2012a, 2012b) influence withanolides production in the plant tissue and organ cultures of W. somnifera (Table 4). An attempt is made here to discuss various factors that have a significant impact on the production of withanolides.

Type of explants

The selection of fast-growing in vitro cultures is an important factor to produce a higher amount of desired products (Smetanska 2008). Sharada and the group analyzed the synthesis of withanolides from different explants of W. somnifera (Sharada et al. 2007). They observed the highest level of withanolides from cultures established from leaf explants in comparison to shoot and root cultures. This could be due to the different morphologies and intrinsic biosynthetic capabilities that produce a wide range of secondary metabolite content. Further, Sivanandhan et al. (2011) recorded that the leaves and roots of in vitro grown cultures of W. somnifera contained higher content of withanolides, withanone, and withaferin A in comparison to stem explant. Sabir et al. (2013) also observed the maximum content of withanolides.
in in vitro shoots of *W. somnifera*, when compared with in vitro root and callus tissues. The differences in withanolides content might be due to the morphogenic transitions and the metabolic switching of withanogenesis is intricately linked to the differentiation-dedifferentiation-regeneration phases that alter the phytochemical composition.

Table 3 Factors influencing callus mediated shoot organogenesis in *W. somnifera*

| Explant                        | Callus induction (Media + PGRs) | Shoot bud induction (Media + PGRs) | Rooting (Media + PGRs) | Reference                        |
|--------------------------------|---------------------------------|-----------------------------------|------------------------|----------------------------------|
| Leaf segments from field-grown plants | MS + 2,4-D (2.0 mg/l) + KN (0.2 mg/l) | MS + 2,4-D (2.0 mg/l) + KN (0.2 mg/l) | MS + IBA (2.0 mg/l) + IAA (2.0 mg/l) | (Rani and Grover 1999) |
|                                | MS + KN (0.5 mg/l) + 2,4-D (2.0 mg/l) | – | – | (Rani et al. 2016) |
| Nodal segments from field-grown plants | MS + BAP (4.5 µM) + KN (1.0 µM) + NAA (0.5 µM) | MS + BAP (9.0 µM) + IAA (1.0 µM) | MS | (De Silva and Senarath 2009) |
| Internodal segments from field grown plants | MS + 2,4-D (2.26 µM) | MS + BAP (4.44 µM) + IAA (0.57 µM) | ½ MS + IBA (9.84 µM) | (Manickam et al. 2000) |
| Shoot apex from field-grown plants | MS + 2,4-D (1.0 mg/l) + BAP (2.0 mg/l) | – | – | (Rani et al. 2016) |
|                                | MS + KN (1.0 µM) + 2,4-D (1.5 µM) + BAP (4.5 µM) | MS + BAP (9.0 µM) + IAA (1.0 µM) | – | (De Silva and Senarath 2009) |
| Axillary shoots                 | MS + 2,4-D (2.0 mg/l) + KN (0.2 mg/l) | MS + BAP (2.0 mg/l) | MS + IBA (2.0 mg/l) + IAA (2.0 mg/l) | (Rani and Grover 1999) |
| Leaf discs from in vitro seedlings | MS + BAP (2 mg/l) + IAA (0.5 mg/l) | MS + BAP (2 mg/l) + IAA (0.5 mg/l) | MS | (Dewir et al. 2010) |
|                                | MS + BAP (1.0 mg/l) + 2,4-D (1.0 mg/l) | MS + BA (2.0 mg/l) + NAA (1.0 mg/l) | MS + IBA (2.0 mg/l) | (Rout et al. 2011) |
|                                | MS + 2,4-D (3.0 mg/l) | MS + BAP (4.0 mg/l) | MS + NAA (10.0 mg/l) | (Arumugam and Gopinath 2013) |
|                                | MS + 2,4-D (3.0 mg/l) | MS + BAP (4.0 mg/l) | MS + NAA (4.0 mg/l) | (Arumugam and Gopinath 2013) |
|                                | MS + 2,4-D (0.5 mg/l) + KN (0.2 mg/l) | MS + BAP (0.5 mg/l) + IBA (0.2 mg/l) | MS + IBA (0.5 mg/l) | (Chakraborty et al. 2013) |
| Hypocotyl from in vitro seedlings | MS + 2,4-D (3.0 mg/l) | MS + BAP (2.0 mg/l) | MS + NAA (5.0 mg/l) | (Arumugam and Gopinath 2013) |
| Epicotyl in vitro seedlings     | MS + 2,4-D (3.0 mg/l) | MS + BAP (4.0 mg/l) | MS + NAA (5.0 mg/l) | (Arumugam and Gopinath 2013) |
| Nodal segments from in vitro seedlings | MS + KN (1.0 mg/l) | MS + KN (1.0 mg/l) | MS + IAA (0.5 mg/l) | (Shukla et al. 2010) |
|                                | MS + NAA (2.0 mg/l) | MS + BAP (2.0 mg/l) | MS + IBA (2.0 mg/l) | (Waman et al. 2011) |
|                                | MS + 2,4-D (2.0 mg/l) + KN (0.2 mg/l) | MS + BAP (1.0 mg/l) + IAA (0.2 mg/l) | MS + IBA (2.0 mg/l) | (Udayakumar et al. 2014) |

*Mostly all these reports used MS medium with 3% sucrose and agar as a gelling agent
MS, Murashige and Skoog (1962) medium; 2,4-D, 2,4 dichlorophenoxyacetic acid; PGRs, plant growth regulators; KN, kinetin; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; BAP, 6-benzylaminopurine; NAA, α-naphthalene acetic acid; GA₃, gibberellic acid
| Culture type          | Explant                  | Media + PGRs                  | Additives                        | Elicitation/treatment | Metabolite extracted                  | Reference                      |
|----------------------|--------------------------|------------------------------|----------------------------------|-----------------------|----------------------------------------|---------------------------------|
| Multiple shoot culture | Shoot tips               | MS (liquid) + BAP (1.0 mg/l)  | Coconut milk (10%)               | –                     | Withaferin A (136 mg/100 g DW)         | (Ray and Jha 2001)              |
| Shoot                | Nodal segments           | MS (liquid) + BAP (0.6 mg/l) + Phytagel (0.2%) | Spermidine (20 mg/l) Salicylic acid (100 µM) | –                     | Withanolides A (8.48 mg/g DW, Withanolides B (15.47 mg/g DW), Withaferin A (29.55 mg/g DW), Withanone (23.44 mg/g DW) | (Sivanandhan et al. 2013) |
|                      |                          | MS + BAP (1.5 mg/l) + IAA (0.3 mg/l) + Sucrose (6%) + Phytagel (0.2%) | L-Glutamine (20 mg/l)          | –                     | Withanolide A (0.75 mg/g DW), Withanolide B (1.08 mg/g DW), Withanone (1.74 Withaferin A (2.05 mg/g DW) | (Sivanandhan et al. 2015) |
| Shoot clusters       | MS (liquid)               | Gracilaria edulis extract (40%) | –                               | –                     | Withanolide A (0.76 mg/g DW), Withanolide B (1.66 mg/g DW), Withaferin A (2.80 mg/g DW), Withanone (2.42 mg/g DW) | (Sivanandhan et al. 2014) |
| Shoot tip and mature node | MS + IAA (0.4 µM) + BAP (0.4 µM) | –                             | –                               | –                     | Withanolide A (2.59 µg/g (d.m.), Withanone (1.61 µg/g (d.m.)) | (Sharada et al. 2007) |
| Axillary buds        | MS + BAP (1.0 mg/l) + KN (1.0 mg/l) | –                             | –                               | –                     | Withanolide A (1.167 mg/gDW)         | (Sabir et al. 2008)            |
| Seed                 | MS + BAP (1.0 mg/l)       | –                             | –                               | –                     | Withaferin A (980 ± 0.97 µg/g DW)     | (Senthil et al. 2015)          |
| Culture type          | Explant                        | Media + PGRs                                  | Additives | Elicitation/treatment | Metabolite extracted                                      | Reference                     |
|-----------------------|--------------------------------|-----------------------------------------------|-----------|-----------------------|-----------------------------------------------------------|-------------------------------|
| Adventitious root     | Leaf segments                  | MS (liquid) + + IBA                           | –         | –                     | Withanolide A (380 ± 0.36 µg/g DW)                         | (Senthil et al. 2015)         |
|                       |                                | (1.0 mg/l) + IAA (0.25 mg/l)                 |           |                       |                                                            |                               |
|                       |                                | MS (liquid) + IBA (1.0 mg/l)                 | –         | –                     | Total Withanolides (1.621 mg/g DW), Withaferin A (1.362 mg/g DW) | (Rangaraju et al. 2019)       |
|                       |                                | MS (liquid) + IAA (0.25 mg/l) + IBA (0.75 mg/l) |           |                       | Total withanolides (0.084 mg/g DW), Withaferin A (0.023 mg/g DW), Withanoside IV (0.061 mg/g DW) | (Rangaraju et al. 2018)       |
|                       |                                | MS (liquid) + IBA (2.0 mg/l) + IAA (0.5 mg/l) |           |                       | Withanolide A (380 µg/g DW), Withaferin A (5.36 µg/g DW) | (Senthil et al. 2015)         |
|                       |                                | ½ MS + IBA (0.5 mg/l)                        |           |                       | Withanolide A (8.8 mg/g DW)                               | (Praveen and Murthy 2010)     |
| Callus derived from   | Leaf segments                  | MS + 2,4-D (2.0 mg/l) + KN (0.2 mg/l) + Sucrose (3%) |           | Salicylic acid (150 µM) | Withanolide A (64.65 mg/g DW), Withanolide B (33.74 mg/g DW), Withaferin A (17.47 mg/g DW), Withanolide B (42.88 mg/g DW), 12-deoxy withastraamolide (5.34 mg/g DW), Withanoside V (7.23 mg/g DW), Withanoside IV (9.45 mg/g DW) | (Sivanandhan et al. 2012a)    |
|                       | segments                       | (for callus induction), ½                     |           |                       |                                                           |                               |
|                       |                                | MS + IBA (0.5 mg/l) + IAA (0.1 mg/l) + Sucrose (2%) |           |                       |                                                           |                               |
|                       |                                | (for adventitious root induction)             |           |                       |                                                           |                               |
| Callus derived from   | Leaf segments                  | MS + 2, 4-D (2.0 mg/l) + KN (0.2 mg/l) + Sucrose (3%) |           | Chitosan (100 mg/l)  | Withanolide A (323.85 mg/g DW), Withanolide B (0.275 mg/g DW), Withaferin A (3.347 mg/g DW), Withanoside V (0.450 mg/g DW), Withanoside IV (0.528 mg/g DW) | (Sivanandhan et al. 2012b)    |
|                       | segments                       | (for callus induction)                        |           |                       |                                                           |                               |
|                       |                                | ½ MS (liquid) + IBA (0.5 mg/l) + NAA (0.1 mg/l) + Sucrose (2%) |           |                       |                                                           |                               |
|                       |                                | (for adventitious root induction)             |           |                       |                                                           |                               |
The extraction of secondary metabolites on a large-scale is possible by manipulating the culture medium (Praveen and Murthy 2010). The ideal culture media should be inexpensive, readily available, easily prepared, and metabolite inducer for secondary metabolites production (VanderMolen et al. 2013). *W. somnifera* was studied for its accomplishments in the synthesis of withanolides under the influence of a basal media (Praveen and Murthy 2010; Ray and Jha 2001). The maximum content of withaferin A (0.09%) was recorded in shoot tips proliferating on B5 medium in comparison with the other types of basal medium (MS, B5, WPM, and SH) (Ray and Jha 2001). Whereas, withanolide A accumulation was higher on MS medium (0.065%) than in B5 medium (0.03%). Similarly, the effect of different types of tissue culture medium (MS, B5, NN, and N6) on the production of withanolides from adventitious root cultures of *W. somnifera* was investigated (Praveen and Murthy 2010). Among the different media, the maximum accumulation of withanolide A was reported on MS medium (8.27 mg/g DW) followed by B5 Medium (6.66 mg/g DW). Furthermore, different strengths (0.25, 0.5, 0.75, 1.0, 1.5, 2.0) of MS medium were investigated to find the optimum nutrient content for the production of withanolide A and ½ MS favoured the higher production of withanolide A in *W. somnifera* (8.65 mg/g DW).

**Growth regulators**

The types and concentrations of PGRs particularly cytokinins affect the synthesis of withanolides in in vitro grown plants of *W. somnifera* (Roja et al. 1991). It was investigated the addition of different cytokinins (BA, Zeatin, KN, 2iP, and TDZ) to the culture medium influence the production of withanolides in multiple shoot cultures of *W. somnifera* (Sivanandhan et al. 2013). Among the different PGRs tested, the maximum increase in withanolide A (6-fold), withanolide B (7.6-fold), withaferin A (1.12-fold), and withanosene (1.16-fold) was recorded in nutrient medium fortified with BA (0.6 mg/l) in comparison with control. Similarly, Ray and Jha (2001) recorded the maximum accumulation of withanolide D and withaferin A from shoot tips in the culture medium with BAP (1.0 mg/l), and a decrease in the content of withanolides was observed with an increased concentration of BAP (2.0 mg/l–5.0 mg/l). Further, adventitious roots obtained from the leaf segments of *W. somnifera* on ½ MS medium enriched with IAA (0.25 mg/l) and IBA (0.75 mg/l) accumulated withaferin A and withanoside IV (Rangaraju et al. 2018). Similarly, adventitious roots obtained from the callus of leaf segments cultured on MS medium containing IBA (0.5 mg/l) and IAA (0.1 mg/l) stimulated the higher accumulation of all withanolides (Sivanandhan et al. 2012a).
Also, the increased accumulation of withaferin A was observed in callus obtained from leaf segments on culture medium enriched with TDZ (0.5 mg/l) and NAA (0.5 mg/l) (Adil et al. 2019).

**Carbon and nitrogen sources**

The addition of carbon and nitrogen sources in the nutrient medium is critical for phytochemical production (Nielsen et al. 2011). In *W. somnifera*, the influence of various types of carbon sources (glucose, sucrose, fructose, and maltose) and nitrogen sources (ammonium nitrate, adenine sulphate, potassium nitrate, sodium nitrate, and L-glutamine) was studied on the synthesis of secondary metabolites (Sivanandhan et al. 2015). They found that the optimized culture medium (MS + 1.5 mg/l BA + 0.3 mg/l IAA) with 6% sucrose and 20 mg/l L-glutamine induced maximum withanolides content (2.05 mg/g DW withaferin A, 0.75 mg/g DW withanolide A, 1.08 mg/g DW withanolide B, and 1.74 mg/g DW withanone). In literature, it was evidenced that nitrogen compounds interact with carbon sources to regulate the expression of pathway genes involved in secondary metabolite production. Similarly, Ray and Jha (2001) noted the higher production of withanolide D in culture medium augmented with a higher concentration (4%) of sucrose. Whereas the maximum content of withaferin A was recorded in a nutrient medium enriched with 8% sucrose.

**Status of the medium**

In recent years, the liquid culture system considered an attractive alternative to a solid medium for the production of important phytochemicals due to the reduction in production costs (Sivanandhan et al. 2013; Pati et al. 2011). The use of a liquid culture system has been documented by many researchers in *W. somnifera* (Mir et al. 2014; Sivanandhan et al. 2013, 2014; Ray and Jha 2001; Senthil et al. 2015; Rangaraju et al. 2019, 2018). Mir et al. (2014) recorded the enhancement in withaferin A content in shoot cultures grown on ½ MS liquid medium with BAP (5.0 µM) after 5 weeks culture. Similarly, a higher accumulation of withanolides was recorded on MS liquid medium containing different PGRs and additives (Sivanandhan et al. 2013, 2014; Ray and Jha 2001).

**Additives**

The effect of growth additives such as coconut milk, yeast extract, casein hydrolysate, and spermidine on withanolides production was documented in *W. somnifera* (Ray and Jha 1991; Sivanandhan et al. 2013). Ray and Jha (1991) investigated the effect of different growth additives such as yeast extract, casein hydrolysate, and coconut milk on withanolides content. They found that the maximum accumulation of withaferin A (136 mg/100 g DW) on optimized MS liquid medium supplemented with coconut milk (10%) after 4 weeks of shoot culture, while the higher content of withanolide D (100 mg/100 g DW) was recorded in casein hydrolysate (500 mg/l). Earlier research demonstrated that polyamines and PGRs imitate secondary metabolite production by activating particular genes in root and shoot cultures. Sivanandhan et al. (2013) found the increased production of withanolides in in vitro grown plants inoculated on a culture medium with PGRs and spermidine (20 mg/l).

**Elicitors**

Elicitation is a technique of inducing or increasing the production of secondary metabolites in plant tissue culture. The influence of different abiotic (salicylic acid, aluminum chloride, and methyl jasmonate) and biotic (chitosan) was investigated on the accumulation of withanolides in *W. somnifera* (Sivanandhan et al. 2012a, 2012b). They found a 48, 37, 29, and 20-fold enhancement of withanolide B, withaferin A, and withanone, respectively on elicitation of adventitious root culture with salicylic acid at the concentration of 150 µM at 10 days exposure time. Further, the enhanced production of withanolides (1.14 to 1.18-fold) was achieved on MS liquid medium enriched with 0.6 mg/l BA and 20 mg/l spermidine with a favourable response to elicitation with salicylic acid (100 µM) at 4 h exposure time (Sivanandhan et al. 2013). Moreover, Sivanandhan and the group investigated the effect of seaweed extracts of *Gracilaria edulis* and *Sargassum wightii* on withanolides production in multiple shoot suspension culture of *W. somnifera*. The maximum content of withanolide B, withaferin A, and withanone was recorded at 24 h exposure time in MS liquid medium augmented with *G. edulis* (40%) extract (Sivanandhan et al. 2014).

**Conclusion**

Due to a major worldwide trend toward phytotherapeutics, there has been a significant increase in the use of medicinal plants for the healthcare industry. Among medicinal plants, *W. somnifera* has gained a lot of interest owing to its enormous pharmacological properties against a large number of diseases. Hence, there is a need for the establishment of economical and feasible strategies for the large-scale propagation of *W. somnifera*. Plant cell and tissue culture have the potential to enable the rapid multiplication of elite cultivars of *W. somnifera* and help the herbal industries to have easy access to raw materials. The use of in vitro grown *W. somnifera* plants for the synthesis of pharmaceutically important constituents offers an alternative source for preclinical applications.
and clinical research. Hence, catalyzing the existing genetic resources through in vitro culture approaches will immensely help in understanding the biosynthesis, transport, accumulation, and modulation of secondary metabolites and will lead to further improvement of this hugely important medicinal plant.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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